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FURTHER OBSERVATIONS ON THE VIRULENCE OF HÆMOLYTIC STREPTOCOCCI, WITH SPECIAL REFERENCE TO THE MORPHOLOGY OF THE COLONIES.*

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SINCE Arkwright (1921) described rough and smooth variants of *B. typhosus* and *B. paratyphosus*, a number of observers have recorded similar variations in cultures of other micro-organisms, and in every case, except anthrax, the relation of these variants to virulence has been the same, the rough form being attenuated, while the smooth type is relatively virulent. Cowan (1922), by growing hæmolytic streptococci alternately in broth and on agar, and by selecting at each operation the broth cultures or colonies which displayed the highest degree of roughness or smoothness, succeeded after several months in dissociating rough and smooth variants which conformed with the general rule that the rough form is attenuated and the smooth form is virulent. The present writer (1927), working on the virulence of hæmolytic streptococci freshly isolated from cases of puerperal septicæmia, found that the cocci became attenuated when cultivated on agar slopes, and that the lost virulence could be restored by cultivation in normal human serum provided that the cocci had not been cultivated on artificial media for a long time. After prolonged cultivation on agar, cultures were obtained which appeared to be permanently attenuated, their virulence being unalterable by cultivation in serum, and by this means two types of the same strain were obtained—(i) a permanently attenuated variety, and (ii) a type which could be exalted by cultivation in serum or attenuated by cultivation on agar. The length of time during which subcultivation had to be continued before the stage of permanent attenuation was reached varied greatly with different strains, but permanently attenuated cultures were ultimately obtained from each of the four strains examined.

Subsequent examination of these two types for rough and smooth characteristics led to the unexpected observation that the permanently attenuated cocci grew in smooth, or as I called them in a previous paper, "glossy" colonies on agar and produced uniform turbidity in broth, while the virulent type formed

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colonies with matt surfaces on agar and produced clumps in broth. All the freshly-isolated cultures examined were entirely composed of matt colonies, but after a few subcultures on agar and in broth glossy colonies began to appear on the plates, and their number gradually increased until the matt colonies had entirely disappeared. This disappearance of matt colonies is possibly aided by the fact that cocci from glossy colonies multiply more rapidly in broth than those derived from matt colonies, so that, after frequent subcultivation, cultures are obtained which contain a preponderance of glossy colonies, and ultimately the matt form is entirely supplanted by its more rapidly-multiplying rival.

Permanently attenuated cultures may be obtained either by picking glossy colonies when they first appear in a recently-isolated culture, or by allowing the matt colonies to disappear naturally after prolonged subcultivation *in vitro*. The glossy type is always attenuated and cannot be exalted by cultivation in serum, but, although the matt form may be equal in its degree of attenuation to the glossy type, yet it differs from the latter in being susceptible to exaltation by cultivation in undiluted normal human serum. It remained to be determined whether animal passage would restore virulence to glossy cultures, or whether their virulence would be as little affected by animal passage as by cultivation in serum *in vitro*. Mouse passage experiments, which will be described in this paper, have confirmed the results obtained *in vitro*, for, although the virulence of attenuated matt cultures is exalted by mouse passage, attempts to increase the virulence of the glossy variants by similar means have failed. When a single matt colony, which is either actually or potentially virulent, is picked from a plate and subcultured daily in broth, periodical plating and examination of colonies reveals sooner or later the reappearance of glossy colonies in the culture, but the reverse process of obtaining matt colonies from glossy colonies has never been observed, and attempts to increase the virulence of the glossy colonies, without necessarily altering their form, have been unsuccessful.

METHOD OF PREPARING A SUITABLE CULTURE MEDIUM FOR THE RECOGNITION OF MATT AND GLOSSY COLONIES.

I am indebted to my colleague, Dr. Fleming, for the following method of preparing a culture medium on which matt and glossy colonies can be easily distinguished, and for bringing to my notice the different characteristics of colonies of virulent and attenuated cultures. Matt and glossy colonies can sometimes be distinguished from each other on blood agar plates after 24 hours' incubation, and they are more easily distinguishable when the plates have been left at room temperature for a few days, but the differences are much more pronounced if the blood agar is heated at 80° C. before pouring the plate. The method which has given the best results is to mix about 1 c.c. of human blood with about 20 c.c. of melted agar at 45° C. in a 7″ × 1″ test-tube, and then to place the test-tube in a water-bath at 80° C. for two minutes, after which the plate is poured. The medium prepared in this way is of a reddish-brown colour, and is superior to ordinary blood agar or boiled blood agar for the differentiation of matt and glossy colonies. If the medium is heated to a temperature higher than 80° C., or if it is heated at 80° C. for longer than two

or three minutes, the advantages of the medium are lost. Fig. 1 shows matt and glossy colonies which had been incubated for 24 hours on the same plate and which were derived from the same strain of hæmolytic streptococcus.

The matt colonies are tough and can be pushed along the surface of the medium without loss of contour, but the glossy colonies are of watery consistence and disintegrate when touched.

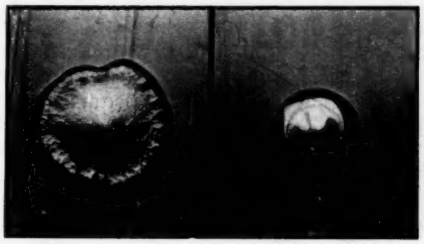

FIG. 1.

ATTEMPT TO INCREASE THE VIRULENCE OF GLOSSY COLONIES BY MOUSE PASSAGE.

The permanently attenuated culture used in this experiment, which was entirely composed of glossy colonies, had been virulent to mice when freshly isolated by blood culture from a case of puerperal septicæmia, and it had become attenuated after repeated subcultivation *in vitro* without any selection of colonies. Before the commencement of this experiment 0·25 c.c. of a 16-hours' broth culture failed to kill a mouse when inoculated intraperitoneally—a dose which was estimated by counts in blood-agar plates to contain 65 million living cocci—but inoculation of 0·5 c.c. caused fatal septicæmia. A mouse was therefore inoculated intraperitoneally with 0·5 c.c. of the culture, and on the following day a broth culture was prepared from the heart's blood of the dead mouse. On the third day this broth culture was passed to a fresh mouse, and by this means the culture was carried from mouse to broth and from broth to mouse until it had passed through nine mice. As there was no evidence of any increase of virulence cultivation in broth was abandoned, the peritoneal cavities of the mice were washed out with broth, and the washings were re-inoculated immediately into fresh mice. When the culture had been passed through 16 mice by this method, a 16-hours' broth culture was prepared from the heart's blood of the last mouse, and falling dilutions (0·25 c.c.) were inoculated intraperitoneally into two series of mice. All the mice were alive and well 14 days after inoculation. The largest dose of the culture was estimated, by counts in blood-agar plates, to contain 24 million living cocci. During the passage of this strain through the peritoneal cavities of 16 mice it had, on four occasions, become contaminated with coliform organisms necessitating four breaks in direct transmission to purify the culture by plating. To avoid contamination from the alimentary canal, passage experiments were commenced in which mice were inoculated by the intravenous route, and the cocci were recovered from the heart's blood after death. As it was found that mice frequently survived intravenous

inoculations of 1 c.c. of undiluted culture, it was necessary to add a thick suspension of killed coliform bacilli to the cocci to secure a fatal septicæmia. The experiment was commenced by inoculating a mouse intravenously with 1 c.c. of the culture, which had already been passed through 25 mice, reinforced with a thick suspension of dead coliform bacilli, and on the following day blood was aspirated from the heart and mixed with 0·5 c.c. of broth. The blood + broth was incubated until hæmolysis was evident; dead coliform bacilli were then added, and the whole mixture was inoculated intravenously into a fresh mouse. Uninterrupted passage from mouse to mouse was impracticable, because direct transfer of the heart's blood of a dead mouse to the circulation of a fresh mouse frequently failed to cause fatal septicæmia, and daily incubation of the heart's blood in broth, until hæmolysis was evident (3 to 7 hours), was therefore necessary.

When the cocci had been passed through 6 mice in this way, a 16-hours' broth culture, prepared from the heart's blood of the last mouse, was tested for virulence by inoculating 0·25 c.c. of falling dilutions of the culture into the peritoneal cavities of two series of mice. Both the mice which received undiluted culture, estimated to contain 90 million living cocci, died within 24 hours, but all the mice receiving smaller numbers of cocci were alive and well 14 days after inoculation. As the minimal lethal dose in this experiment contained a larger number of living cocci than any of the doses which failed to kill mice in the earlier tests, there is no evidence that passage through 31 mice altered the virulence of the cocci. During the course of this experiment the cocci were plated out daily, but no evidence of reversion to the matt form was obtained.

EXALTATION OF VIRULENCE OF ATTENUATED MATT COLONIES BY MOUSE PASSAGE.

The culture used in this experiment, which was derived from the same original blood culture as the permanently attenuated cocci considered in the preceding paragraph, had become attenuated by cultivation on artificial media, but the natural process of degeneration to a culture composed entirely of glossy colonies had been avoided by picking matt colonies from plates and by subculturing these colonies in broth, so that only a small number of glossy colonies appeared in the culture. By this means an attenuated culture composed entirely of matt colonies was obtained, and its virulence was tested by inoculating 0·25 c.c. of falling dilutions of the culture into the peritoneal cavities of mice. All the mice were alive and well 14 days after inoculation. The culture was passed through 8 mice by direct transfer of peritoneal washings, the sequence being broken once by plating the culture to remove a few contaminating coliform bacilli, and the cocci were then tested for virulence in the usual way. A separate passage experiment, commencing with the same matt attenuated culture, was carried out by the intravenous method previously described, and the culture, which in this case was reinforced by the addition of dead coliform bacilli, was carried through 8 mice without contamination. Table I, which gives the results of these two experiments, shows that in both cases the virulence of the attenuated matt culture was increased by mouse passage.

TABLE I.—*Exaltation of Virulence of Attenuated Matt Culture by Mouse Passage.*

	Dilution of cultures.						
	1 in 1.	1 in 10.	1 in 100.	1 in 1000.	1 in 10,000.	1 in 100,000.	
Attenuated matt culture	**S**	**S**	**S**	**S**	**S**	**S**	Estimated maximum dose of living cocci, 56 million.
The same matt culture after direct intraperitoneal passage through 8 mice	1	**S**	1	2	2	**S**	Estimated maximum dose of living cocci, 6 million.
The same matt culture after intravenous passage through 8 mice	1	2	2	**S**	3	**S**	Estimated maximum dose of living cocci, 38 million.

Each square in the table represents a mouse. Each mouse received 0·25 c.c. of the cultures intraperitoneally in the dilutions indicated. The numbers in the squares give the days on which the mice died. **S** indicates that the mouse was alive and well 14 days after inoculation. The diluent was broth in all experiments.

The results of this experiment are not altogether satisfactory owing to the abnormally high resistance of some of the mice to infection with hæmolytic streptococci—a phenomenon which is frequently observed when a series of mice are inoculated intraperitoneally with diminishing numbers of virulent cocci, but it is evident that the virulence of the culture was exalted by mouse passage.

SUMMARY AND CONCLUSIONS.

Hæmolytic streptococci, freshly isolated by blood culture from cases of septicæmia, when plated out on a special medium, form colonies with matt surfaces, and this is the only type of colony seen on the plate.

If the culture is plated out again, after daily subcultivation on agar for a period varying from a few days to a few months, matt colonies will still be found on the plate, and in addition a different type of colony is seen which can be recognized by its glossy appearance. Experiments ~~are quoted to~~ show that the cocci which form these glossy colonies are permanently attenuated, and cannot have their virulence increased *in vitro* or *in vivo*.

Prolonged subcultivation of a virulent culture on agar causes the disappearance of matt colonies by a natural process of degeneration without any artificial selection, and when this has happened, the culture, which then contains only glossy colonies, is permanently attenuated.

A culture composed of matt colonies may be either virulent or attenuated, but it is always potentially virulent because it can be easily exalted *in vivo* or *in vitro*.

The conclusions arrived at in this paper are supported by observations *in vitro* on four strains of hæmolytic streptococci, and by a series of observations on one strain *in vivo*.

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SEROLOGICAL STUDIES IN THE GROUP OF THE SPORE-BEARING ANAËROBES. I. THE QUALITATIVE ANALYSIS OF THE BACTERIAL ANTIGENS OF *B. ŒDEMATIS MALIGNI* (*VIBRION SEPTIQUE*) AND *B. TETANI*.

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THE method of the "qualitative receptor analysis" was inaugurated by Weil and Felix (1917, 1918,[1,2]) and Weil, Felix and Mitzenmacher (1918), and first extended by Sachs (1918), Braun (1918), Joetten (1919), and Braun and Schäffer (1919); it revealed the fact that every bacterial species so far investigated contains a "stable (O)" antigen, and that in addition certain kinds possess also the "labile (H)" antigen. This has been found to be true in certain species pathogenic for man as well as in others which are saprophytic.

It would lead too far to give here a review of all the facts which are now established and to consider the means by which these results were obtained, or to mention the earlier observations now shown to be included in and explained by this theory.

As far as the agglutination reaction is concerned, it may be stated briefly that the two kinds of antigen and their corresponding antibodies show very distinct and constant differences in many respects. Moreover, these differences are such that the analysis may be considered to be a qualitative one.

The labile antigen is destroyed by heating to 100°C., by treatment with alcohol, dilute acid or dilute alkali, and may occasionally disappear as a normal constituent of the bacillary body. This loss of the labile component of the bacillary substance may occur under natural conditions in the infected host as well as on artificial culture media, leading to the "O" culture as

contrasted with the "H" culture, which possesses both the "O" and the "H" components. The stable antigen, however, is completely resistant to all the different influences mentioned. On the other hand, the two kinds of agglutinins corresponding respectively with the stable and labile antigens also show different physical and chemical properties. The nomenclature suggested by Schiff (1922) has been adopted: stabilotropic for the "O" agglutinin which reacts with the stable "O" antigen, and labilotropic for the "H" agglutinin which combines with the labile antigen. The behaviour of the two agglutinins is the exact opposite of that of the corresponding antigens; the stabilotropic "O" agglutinin is less resistant to the action of heat (Weil and Felix, 1917) and of certain chemical reagents (Felix and Olitzki, in the press) than the "H" agglutinin.

Now from the theoretical point of view the evidence which has been collected during the last few years from the qualitative receptor analysis in the case of *B. proteus* and *B. typhosus* and certain salmonellas may be summarized in the following scheme:

labile antigen	——→ Agglutinin	←——	stable antigen
=H receptor	——→ Precipitin	←——	
	Complement binding immune body	←——	= O receptor
	Bactericidal immune body	←——	
	Opsonin	←——	

This scheme is an attempt to formulate a general conception of the bacterial immune bodies. It illustrates the principles established by Weil and Felix (1920), Felix (1924), and Felix and Olitzki (1926), that the effective agent in complement-fixation as well as in bactericidal serum action consists purely in the interaction of the stable "O" antigen and the "O" immune body, and Braun and Nodake (1924) proved the same to be true in the opsonic serum effect; on the other hand, in agglutination and in precipitation both kinds of antigen and antibody develop their action and effect independently of one another. The different physical and chemical properties of the two kinds of antigen mentioned above seem therefore to find their correlates in their different biological functions.

One serum body, and one only, namely, the stabilotropic immune body, seems to be involved as the sole important factor in those phenomena of humoral immunity specified above, while the biological function of the labile antigen and its serum counterpart is not yet in any way clearly apprehended, in spite of the light thrown on this point by Braun and his co-workers.

This hypothesis will of course meet with much criticism. It was to extend our knowledge concerning the problems stated above that the present investigation of the spore-bearing anaërobes was begun.

SECTION 1.—THE OCCURRENCE OF THE "H" (LABILE) AND "O" (STABLE) ANTIGEN IN *B. ŒDEMATIS MALIGNI* AND *B. TETANI*, DEMONSTRATED BY MEANS OF THE AGGLUTINATION REACTION.

It was to be expected that the two species chosen would show the two types of antigen upon the analogy of the aërobic organisms, in which it is now agreed by all workers that there is a close correlation between the presence of

flagella and of the two kinds of antigen. And as a matter of fact the labile antigen was easily demonstrated. It is true that considerable difficulties are met with in the anaërobes in agglutination tests owing to the instability of normal saline emulsions, but thanks to the method devised by Arkwright (1921) in his work on " rough " cultures of using reduced saline concentrations as low as 0·1% of saline, they were readily overcome.

In the analysis of the antigens, the three essential types of experiment, namely, the agglutination test, the absorption of agglutinins and the production of agglutinins in rabbits were made use of, as had already been done by Weil and Felix and their collaborators in their work upon different aërobes. In the case of *Vibrion septique* there were available certain rabbit and sheep immune sera, which had been prepared and used nearly nine years ago by M. Robertson (1920) in her serological investigation of this species.

The relatively high titre of agglutination still shown by these old sera is due to the labilotropic " H " immune body only. The labile antigen was found to be destroyed by heating to 100°C. for two hours in exactly the same way as in the case of the aërobes. As regards *B. tetani*, two monovalent rabbit sera six months old kindly placed at our disposal by Dr. Schütze were examined, and here also the agglutinins were of the large-flaking labilotropic type.

The resemblance to the large-flaking " H " agglutination already known in *B. proteus* and the typhoid-paratyphoid group was shown in the rapidity of the agglutination, in the large loose appearance of the flakes, which sediment very quickly, and in the nature of the final agglutinate. This persistence of the labilotropic " H " agglutinins in old sera in these two anaërobic species recalls very strikingly the similar conditions obtaining in old sera made with those aërobes which possess the double type of antigen.

These old sera were also investigated for the presence of agglutinins by means of absorption tests. In both species, if the sera were absorbed with bacilli heated to 100°C., or with bacilli treated with alcohol or with normal HCl, the large-flaking " H " agglutinin content remained unaltered, but when the same sera were absorbed with living untreated bacilli the " H " agglutinins were removed.

It may be noted that in destroying the labile antigen by treating the bacilli with alcohol care must be taken to ensure an adequate exposure of the bacillary bodies to the action of the alcohol. The effect of the alcohol may sometimes be reduced by the rapid coagulation of the bacteria into clumps, which include undamaged individuals protected by the envelope of coagulated bacillary bodies. In the re-emulsification these undamaged bacteria are set free, and are then able to react with the sera by means of their persisting labile antigen.

In *B. tetani* it was noted that bacilli heated to 60° C. for two hours show no weakening of their power to absorb the labilotropic agglutinin, while their sensitiveness in flocculation in the simple agglutination test is as a rule definitely impaired.

To reveal the full difference in the properties of the two types of antigen it was necessary to prepare fresh sera so as also to get a pure small-flaking serum. The greatest care was taken to test every individual animal for

normal agglutinins before beginning the immunization. This is particularly necessary in the case of the small-flaking agglutinin, as experience has already shown in the aërobes (Schiff, 1922) that the normal antibodies in many species of animals are of this type. In no instance in a serum dilution of 1 in 10 did any of the rabbits show any agglutination when tested against all the strains of *Vibrion septique* and *B. tetani* used in these experiments.

TABLE I.—Vibrion septique. *Pure Stabilotropic (O) Sera and Mixed Stabilotropic and Labilotropic (H) Sera. Agglutination Test.*

Emulsions of bacilli from glucose broth, washed twice in distilled water. Heated to 100° C. in distilled water (autoclave without pressure for 2 hours). After treatment with alcohol (at room temperature), washed in distilled water three times. All the sediments finally resuspended in saline of the concentration given in the table.

Emulsions agglutinated.	Serum dilutions.	Emulsions of strain "Pasteur III."			Emulsions of strain "Milk IV."		
		Immune sera.		Saline control 0·21% NaCl.	Immune sera.		Saline control 0·21% NaCl.
		51 II "Pasteur" 100°	52 II "Pasteur" 60°.		57 II "Milk" 100°.	58 II "Milk" 60°.	
Living	1:25	++ s	+++	−	± s	+++	−
	1:50	+ s	+++	...	−	+++	...
	1:100	± s	+++	...	−	++±	...
	1:200	−	+++	...	−	++±	...
	1:500	−	++±	...	−	++	...
	1:1000	−	++±	...	...	++	...
	1:2000	−	++±	...	...	+	...
	1:5000	...	++	...	..	±	...
	1:10,000	...	+	...	...	−	...
	1:20,000	...	±	...	...	−	...
	1:50,000	...	(±)	...	...	...	...
	1:100,000	...	−	...	...	...	...
Heated 2 hours to 100° C.	1:25	+++ s	+++ s	−	+ s	++ s	−
	1:50	+++ s	+++ s	...	± s	+ s	...
	1:100	+± s	++± s	...	−	± s	...
	1:200	± s	++ s	...	−	−	...
	1:500	−	+ s	...	−	−	...
Treated with alcohol for 16 hours	1:25	+++ s	+++ s	−	+ s	++ s	−
	1:50	+++ s	+++ s	...	± s	+ s	...
	1:100	+± s	++± s	...	−	± s	...
	1:200	± s	++ s	...	−	−	...
	1:500	−	+ s	...	−	−	...

Total volume, 1 c.c. Reading after 4 hours; 2 hours' incubation at 37° C., then 2 hours at room temperature.

+ + +, + +, +, ± = varying degrees of agglutination, estimated with the naked eye. (±) = traces, estimated by means of a magnifying lens. s = small flakes (stabilotropic). l = large flakes (labilotropic). l + s = large and small flakes.

Table I shows for two strains of *Vibrion septique* placed by M. Robertson in two different agglutination groups (strain Pasteur = Group I, strain milk = Group II), that where antigens were used for immunization in which the labile element was not destroyed, the immune serum showed both agglutinins,

whereas if the labile substance in the antigen was destroyed, the immune serum was found to contain the small-flaking "O" agglutinin only. The immune sera were taken as a rule after four intravenous injections of the particular antigen. In every experiment cited the normal serum of each individual rabbit which had been taken before the immunization was started was put up as a control. These normal sera were in every case found to be negative in the reaction. They are not shown in the tables published.

For convenience in the following account we use the abbreviation 60° immune sera and 100° immune sera for the sera prepared respectively by the injection of bacillary antigen heated to 60° C. for one hour and to 100° C. for two hours.

TABLE II.—B. tetani. *Immunization of Rabbits with One H Culture and One O Culture. Agglutination Test.*

All rabbits bled after 4 intravenous injections.

Agglutination with living emulsions of *B. tetani.*	Serum dilutions.	Immune sera.						Saline control 0·1% NaCl.
		151 II "46" 100°.	152 II "46" 60°.	153 II "46" filtered toxin 100°.	155 II "52" 100°.	156 II "52" 60°.	157 II "52" filtered toxin 100°.	
Strain "46"	1:10	+±s	+++	++s	+s	++s	+s	−
	1:25	+s	+++	+±s	±s	+±s	±s	...
	1:50	±s	+++	±s	±s	±s	(±)s	...
	1:100	−	+++	−	−	−	−	...
	1:200	−	++±	−	−	−	−	...
	1:500	...	++±	...	...	...	...	...
	1:1000	...	++±	...	...	...	...	...
	1:2000	...	+	...	..	...	...	...
	1:5000	...	±	...	...	...	...	...
	1:10,000	...	−	...	...	...	...	...
Strain "52"	1:10	+s	+s	+s	++s	++s	+s	−
	1:25	±s	+s	±s	+±s	+±s	±s	...
	1:50	−	±s	−	+s	+s	−	...
	1:100	−	−	−	−	−	−	...
	1:200	−	−	−	−	−	−	...

In the case of *B. tetani* we were able from the beginning to work with a non-motile culture, which proved, as can be seen in Table II, to be a true "O" form. We owe this strain to the kindness of Dr. Schütze, who passed it on to us along with other strains of *B. tetani.* The "O" culture (No. 52) was isolated by Tenbroeck and Bauer in 1924 from the fæces of a man in Peking. In our hands it showed itself to be toxic, as it was also when tested by Tenbroeck (Tenbroeck and Bauer, 1922).

As is well known, the most striking evidence of the labile nature of the labile "H" antigen is the occurrence of the "O" form, which has lost this antigen, as a mutant under ordinary conditions of culture or in the living host. We hoped to isolate these "O" forms also in the course of this investigation. In *Vibrion septique* we have not yet succeeded in doing this, but in the case of *B. tetani* the existing non-motile strains afforded us this pure "O" reagent.

The pure "O" form of *B. tetani*, it may be mentioned, grows in separate discrete colonies, even on moist media; the "H" form shows a spreading film-like growth even when colonies are also formed. This difference in the character of the growth is quite parallel to that already known in *B. proteus*.

In Table II immune sera produced from strain 52 ("O" culture) and from strain 46 ("H" culture) of *B. tetani* are tested in agglutination. For both strains sera were prepared with bacilli heated to 60° C. and to 100° C.

TABLE III.—Vibrion septique. *Absorption of Agglutinins in One O Serum and One Mixed H and O Serum.*

Emulsions used for absorption: from glucose broth, treated as those for agglutination. Absorption and agglutination in saline of 0·42% NaCl.

Emulsions of strain "Pasteur III" agglutinated.	Serum dilutions.	Serum 52 II—"Pasteur" 60°.			Serum 51 II—"Pasteur" 100°.		Saline control 0·42% NaCl.
		Absorbed in dilution 1:10 with—		Unabsorbed.	Absorbed in dilution 1:10 with "Pasteur" 100°.	Unabsorbed.	
		"Pasteur" living.	"Pasteur" 100°.				
Living	1:10	++± l	++± l	+++	−	++ s	−
	1:25	++± l	++± l	+++	−	++ s	...
	1:50	++ l	++± l	+++ l+s	−	+ s	...
	1:100	+ l	++± l	+++	−	± s	...
	1:200	± l	++± l	+++	−	−	...
	1:500	−	++± l	++±	−	−	...
	1:1000	−	++± l	++±	...	...	...
	1:2000	−	++± l	++±	...	...	...
	1:5000	−	++ l	++ l	...	...	...
	1:10,000	−	+ l	+	...	...	...
	1:20,000	−	± l	±	...	...	...
	1:50,000	−	(±) l	(±)	...	...	...
	1:100,000	−	−	−	...	...	...
Heated 2 hours to 100° C.	1:10	−	−	+++ s	−	+++ s	−
	1:25	−	−	+++ s	−	+++ s	...
	1:50	−	−	+++ s	−	+++ s	...
	1:100	−	−	++± s	−	+± s	...
	1:200	−	−	++ s	−	± s	...
	1:500	−	−	+ s	−	− s	...
	1:1000	−	−	−	...	...	...

respectively, and in addition sera were also made by the injection of bacteria-free filtrates of eight days' growth in glucose broth, heated before injection to 100° C. for two hours. The difference in the action of the sera produced with the bacillary antigen heated to 60° C. for one hour in the case of these two cultures is very striking. The result shows clearly that the serum No. 152 II. against the strain No. 46 had a high, large-flaking agglutination titre, and in addition it contained a low titre of small-flaking "O" agglutinins, corresponding closely to that evoked in sera 151 II and 153 II by the injection of the stable "O" antigen only.

But in strain No. 52 the serum 156 II produced with antigen heated to 60° C. only is quite similar in its action to serum 155 II and 157 II, which were made with antigen heated to 100° C.

We would here draw attention in passing to the "O" agglutination titre of sera 153 II and 157 II produced by bacteria-free filtrates (toxin) heated to 100° C., and we propose to return later to the consideration of this fact.

TABLE IV.—Vibrion septique. *Complement-fixation. Homologous and Heterologous Sera.*

Antigen P = Strain "Pasteur III" = *Vibrion septique*—Group I.
Antigen M = Strain "Milk IV" = *Vibrion septique*—Group II.

Complement-fixation.	Amount of serum.	Immune sera.			
		51 III P. 100°.	52 IV P. 60°.	57 IV M. 100°.	58 IV M. 60°.
Antigen P heated to 56° C.	0·1	±	−	+++	+++
	0·04	+++	++±	+++	+++
	0·02	+++	+++	+++	+++
	0·01	+	+++	+++	++±
	0·005	−	+	+±	+
	0·002	−	−	−	−
Antigen M heated to 56° C.	0·1	+++	++±	+++	−
	0·04	+++	+++	+++	++±
	0·02	+±	+++	+++	+++
	0·01	±	+±	+++	+++
	0·005	−	−	+±	+
	0·002	−	−	−	−
Agglutination.					
Antigen P living	H-titre	0	20,000	0	200
	O-titre	50	50	50	50
Antigen M living	H-titre	0	200	0	5000
	O-titre	50	50	50	50

Complement-fixation:
Total volume 2 c.c.
Serum dilutions + antigen + complement = 1 c.c.
Sensitized sheep red cells 5% = 1 c.c.
4·5 sensitizing doses of amboceptor.
Complement: 0·05 c.c. guinea-pig serum.
Hæmolytic system added after 1 hour at 37° C.
Readings after 1½ hours; half an hour at 37° C. and 1 hour at room temperature (checked by overnight result).
Titre O = a negative result in dilution of 1 : 10.
In this and the following tables antigens and sera in twice the amount used in the test were put up as controls, and always gave complete hæmolysis.

In Table III absorption tests with one 100° serum and one 60° serum are put up side by side. They show that, whereas in both sera the small-flaking agglutinins are completely removed by absorption with the homologous bacilli

heated to 100° for two hours, the large-flaking agglutination titre of serum 52 II (the 60° serum) remained quantitatively unaltered.

These experiments seem to us to show conclusively that the two kinds of antigen are present in these two anaërobic species, and that their properties, as far as they have been investigated up to the present, are analogous to those already known in the aërobes.

TABLE V.—Vibrion septique. *Complement-fixation with Homologous Sera, Absorbed and Unabsorbed.*

Complement-fixation.		Serum 51 II—"Pasteur" 100°.			Serum 52 II—"Pasteur" 60°.		
		Absorbed in dilution 1:10 with—		Unabsorbed.	Absorbed in dilution 1:10 with—		Unabsorbed.
		"Pasteur" 100°.	"Milk" 100°.		"Pasteur" 100°.	"Milk" 60°.	
Antigen P heated 1 hour to 56° C.	Titre	0	0	200	0	0	500
Antigen P heated 2 hours to 100° C.	Titre	0	0	200	0	0	1000
Agglutination.							
Antigen P heated 2 hours to 100° C.	O-titre	0	0	200	0	0	500
Antigen P living	O-titre	0	0	100	0	0	200
	H-titre	0	0	0	20,000	1000	20,000

Titre 0 = a negative result in dilution of 1:25.

Note.—The antigen P heated to 56° C. for 1 hour, used in complement-fixation, was tested for absorption of agglutinins and was found to be active in removing labilotropic (H) agglutinins.

SECTION 2.—THE BEARING OF THE TWO KINDS OF ANTIGEN AND ANTIBODY ON COMPLEMENT-FIXATION.

Our next task was to examine the bearing of the two types of antigen and antibody on the individual immune reactions as set out in the hypothetical scheme. Up to the present we have only investigated the relations which are obtaining in the complement-fixation reaction for both *B. œdematis maligni* (*Vibrion septique*) and *B. tetani*.

In several aërobic species (*B. proteus, B. typhosus, B. enteritidis* (Gaertner) and other salmonellas) it has been definitely established by Weil and Felix (1920) and Weil (1921) that the complement-binding immune bodies as well as the small-flaking agglutinins owe their existence to the stable "O" antigen. While these results have been confirmed to a certain extent, they have not as yet been accepted in their entirety (White, 1926; Braun and his collaborators; Hofmeyer, 1927; Springut, 1927).

It seemed, therefore, of quite particular interest to examine the relations obtaining in the matter of complement-fixation in so widely differing a group

as that of the spore-bearing anaërobes. In the sera already mentioned, one part of which contained only the stabilotropic immune body and the other part which contained both the labilotropic and stabilotropic immune bodies, we had at our disposal the necessary reagents for this purpose.

Table IV illustrates the behaviour of four immune sera against two strains of *Vibrion septique* belonging to two different agglutination groups, in complement-fixation and in agglutination. The normal sera of the four

TABLE VI.—B. tetani. *Complement-fixation. Homologous and Heterologous Sera.*

Antigen "46" = Strain "46" = Group V.
Antigen "52" = Strain "52" = Group VI. } (Tenbroeck and Bauer).

Complement-fixation.	Amount of serum.	*B. tetani* immune sera.					
		151 II "46" 100°.	152 II "46" 60°.	153 II "46" toxin 100°.	155 II "52" 100°.	156 II "52" 60°.	157 II "52" toxin 100°.
Antigen "46" heated to 56°C.	0·1	+++	+++	+	+++	+++	−
	0·04	++±	+++	+±	+++	+++	±
	0·02	+	+++	+	+++	+++	+++
	0·01	±	+++	±	+++	+++	+++
	0·005	−	+++	−	+++	+	+±
Antigen "52" heated to 56°C.	0·1	±	+	±	+++	+++	−
	0·04	±	+++	±	+++	+++	±
	0·02	−	+++	−	+++	+++	−
	0·01	−	++±	−	+++	+++	−
	0·005	−	±	−	+++	++±	−
Agglutination.							
Strain "46" living	H-titre	0	5000	0	0	0	0
	O-titre	50	100	50	50	50	25
Strain "52" living	H-titre	0	0	0	0	0	0
	O-titre	25	50	25	50	50	25

Titre 0 = a negative result in dilution of 1 : 10.

rabbits used for immunization, which were taken before the injections were begun, were examined simultaneously as controls, but are not included in the Table. They were entirely inactive. It is evident that there is a complete parallelism between small-flaking agglutination and complement-fixation, but that there is on the other hand a striking discrepancy between large-flaking agglutination and complement-fixation.

In Table IV it has been shown that the sera act in complement-fixation in accordance with the ratio of their content of small-flaking "O" agglutinins. Table V demonstrates that by the absorption of the sera (which contain both agglutinins) with "O" antigen (antigen heated to 100°), they are deprived at one and the same time of both the capacity to bind complement and to cause

small-flaking agglutination; this is equally true for serum 51 II, which contained only "O" agglutinins to a titre of 1 in 200, and for serum 52 II, which contained an approximately equal amount of "O" agglutinin and in addition "H" agglutinins to a titre of 1 in 20,000.

The contrast between the activity in complement-fixation of the apparently small content of stabilotropic antibody as against the indifference in this reaction

TABLE VII.—B. tetani. *Complement-fixation and Agglutination with Homologous Serum, Absorbed and Unabsorbed.*

Absorption with two strains of *B. tetani* ("46" and "52"), two strains of *Vibrion septique* ("Pasteur" and "Milk"), and one strain of *B. typhosus*.

Complement-fixation.	Amount of serum.	Serum 152 II—*B. tetani* "46" 60°.					
		Absorbed in dilution 1 : 10 with—					Unabsorbed.
		"46" 100°.	"52" 100°.	"Pasteur" 100°.	"Milk" 100°.	*B. typhosus.* 100°.	
Antigen "46" heated 1 hour to 56° C.	0·02	+	++±	+++	+++	+++	+++
	0·01	−	+	++±	++±	++±	++±
	0·005	−	−	+	+	+	+
	0·002	−	−	−	−	−	−
Antigen "52" heated 1 hour to 56° C.	0·02	−	−	+++	+++	+++	+++
	0·01	−	−	++±	++±	++±	++±
	0·005	−	−	−	−	−	−
	0·002	−	−	−	−	−	−
Agglutination.							
Strain "52" living.	0·04	(±) s	+± s	+± s	+± s	+± s	+± s
	0·02	−	(±) s	± s	± s	± s	± s
	0·01	−	−	(±) s	(±) s	(±) s	(±) s
	0·005	−	−	−	−	−	−
Strain "46" living	0·005	++± l	++± l	++± l	++± l	++± l	++± l
	0·002	++± l	++± l	++± l	++± l	++± l	++± l
	0·001	++ l	++ l	++ l	++ l	++ l	++ l
	0·0005	+ l	+ l	+ l	+ l	+ l	+ l
	0·0002	± l	± l	± l	± l	± l	± l
	0·0001	−	−	−	−	−	−

of the presence of "H" labilotropic antibody (in an amount 100 times as great) is very clearly brought out in Tables IV and V. Table V also shows that the value of the antigen used in complement-fixation is independent of the presence or absence of the labile element.

Tables VI and VII show that the same conditions prevail in the case of *B. tetani* as have just been discussed for *Vibrion septique*. The normal sera of the individual rabbits taken prior to the immunization were again used as controls, and also three immune sera against *Vibrion septique* which had shown

marked complement-fixation with their homologous antigen. All these control sera were inactive in complement-fixation with the two tetanus antigens. They are omitted from the tables. The six tetanus immune sera used again showed the independence of complement-binding immune body and labilotropic agglutinin, and the two antigens used, one of which was a pure "O" antigen of a natural "O" culture (strain 52) and the other a normal "H" culture (strain 46), showed that the "H" antigen is not involved in this reaction.

In the absorption test illustrated in Table VII a number of different controls were introduced to exclude the interference of other than specific factors in the experiment. They were not included in the table for reasons of economy in reproduction. The sera were absorbed with heterologous antigens (two strains of *Vibrion septique* and a strain of *B. typhosus*) without in any way altering the degree of complement-fixation and the small-flaking agglutination. A further control which is also omitted from the table (VII) was made by testing the effect of "extract in serum" of the absorbing antigens on complement-fixation and small-flaking agglutination. No interference in either reaction was revealed, and this possible source of error could therefore be excluded in this test.

The results shown in Table VII again emphasize the purely stabilotropic nature of the complement-binding immune body.

DISCUSSION.

The recognition of the double type of antigen would, it was considered, throw light on the relations of species and sub-species in the group of the spore-bearing anaërobes. Data of this type had already proved to be of great value in elucidating the interconnections in several aërobic groups (Weil and Felix, 1918[1], 1920; Schiff, 1922; White, 1925, 1926; Olitzki, 1926).

In regard to *Vibrion septique* serological groups were recognized by Robertson (1920) and in *B. tetani* by Tulloch (1917), and this work was subsequently extended by Tenbroeck and Bauer (1922) and by Fildes (1925). This serological grouping was in both cases done by means of the agglutination reaction, and as a result four groups in *Vibrion septique* and seven in tetanus have been described up to the present.

Now in sera prepared in the ordinary way, by immunization with antigen in which the labile element has not been destroyed, the labilotropic immune body always very greatly exceeds the stabilotropic one, the proportion being about 50 to 100 times as great or even more. We could therefore conclude that the grouping used by the previous workers was based on the estimation of the action of the labilotropic agglutinin only, the low titre of the accompanying stabilotropic "O" agglutination having been overlooked. Tulloch and Robertson founded their idea of the serological limitation of the respective species on the identity of the toxin-antitoxin reaction, and considered that the agglutination reaction was ultra-specific. Our results so far show that there is considerable overlapping of the different agglutination groups as revealed by absorption of agglutinins, and that in the case of the stable antigen the group relationship is very close indeed. If this relationship is examined by

means of complement-fixation the degree of the community of the stable antigen is very strikingly brought out. This is in accordance with many observations among certain groups of aërobes, notable in *B. typhosus*, *B. enteritidis* and other salmonellas, and in *B. proteus*.

We have now to consider very briefly the place of the soluble toxin in the hypothetical scheme, in which it has not, so far, been included. Our work in this direction is still in the earliest stages and is incomplete, but it already appears that the antitoxin holds a place independent of the elements upon which the arrangement of the scheme is based, namely the two bacillary antigens "O" and "H." This is in agreement with a good deal of previous work. It must be emphasized, however, that, as is already known, the broth filtrate, the usual medium containing the toxin, is not free from these elements. This explains why antitoxic sera may show antibodies reacting in those reactions which are connected with the "O" and "H" antigens in addition to their antitoxin content. Thus both rabbit and sheep sera produced by the injection of bacteria-free filtrates of *Vibrion septique* showed a high titre of labilotropic "H" agglutinins, and on the other hand in the case of *B. tetani* and *Vibrion septique*, the intensive immunization of rabbits with broth filtrates heated to 100° C. for two hours produced sera with a content of stabilotropic "O" antibodies showing small-flaking "O" agglutination and complement-fixation in about the same degree as sera made with the heated bacillary bodies as antigen. But no parallelism at all has ever been found between the intensity of these reactions and the antitoxic value of the serum.

Dean (1927), in his recent paper on complement-fixation in mixtures of diphtheria toxin and antitoxin, while able to demonstrate, in spite of considerable technical difficulties, an incomplete and transitory complement-fixation, is, to quote his own words, "unable to prove that this complement-fixation depends on the interaction of the actual toxin and antitoxin."

It remains as a task for future research to determine if the independent substance, which we designate with the word "toxin," contains in its turn a labile and a stable fraction, as is the case in the antigenic substance of the bacillary body. We feel that it is premature to discuss the interesting papers of Tenbroeck and Bauer (1925, 1926), which indicate a type specific anti-bacterial action in immunity against tetanus independent of the toxin-antitoxin relation—a point already raised by Tulloch (1920)—but we intend to go into this question on a later occasion.

SUMMARY.

A. Agglutination.

1. The existence of the two kinds of antigen (O=stable; H=labile) in *B. œdematis maligni* and *B. tetani* has been demonstrated by agglutination, agglutinin-absorption and immunization.

2. The physical and chemical properties of the two kinds of antigen are analogous to those known in those aërobic species which possess the double type of antigen.

3. In *B. tetani* the pure "O" culture (strain 52 from Tenbroeck and Bauer) affords further analogy in cultural respects with *B. proteus*.

B. Complement-fixation.

1. It makes no difference to the occurrence or the intensity of complement-binding whether the bacillary antigen used in the reaction contains both elements or only the stable one. This is equally true when "H" cultures are used heated to 56° C. or to 100° and when the true "O" culture is used (in *B. tetani*).

2. Purely stabilotropic "O" immune sera have strong complement-binding properties.

3. Immune sera which contain both labilotropic and stabilotropic agglutinins do not develop a stronger complement-binding action than "O" immune sera with an equal content of stabilotropic agglutinins; it does not alter the degree of complement-fixation whether "H" agglutinins, even in very great quantities, are present or not.

4. Immune sera which contain both "H" and "O" agglutinins lose entirely their power of binding complement if the "O" agglutinins are removed (by absorption with bacilli heated to 100°), in spite of the fact that the content of "H" agglutinins remains completely unchanged.

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EXPERIMENTS ON THE VIRUS OF FOWL PLAGUE. (I)

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THE investigation of diseases due to the so-called ultramicroscopic viruses presents a series of difficulties which are not encountered in studying bacterial infections where the behaviour of the causal agent can be observed under the microscope or by culture on artificial media.

In dealing with these viruses the only means of determining their presence or absence is by the inoculation of susceptible animals but unfortunately, in the case of a large number of the virus diseases, the usual small experimental animals are either not susceptible to the infection or contract it in so mild a form as to render their use unsatisfactory. A disease to which these objections do not apply is fowl plague, which may be regarded not only as a typical example of an acute infection due to an ultramicroscopic virus but also in many respects as an ideal subject for laboratory study. This has been recognized for many years on the Continent where a great deal of work has been done on the subject but curiously the disease appears to have attracted very little attention in England.

The special advantages of fowl plague as a subject for the study of the ultramicroscopic viruses were emphasized by Schweitzer (1922) and may be briefly stated as follows:—

1. Fowl plague can be taken as a typical representative of the ultramicroscopic viruses, as shown by the ease with which the virus passes all forms of porous filters, its resistance to 50% glycerine, the presence of "inclusion bodies" in the brain-cells, etc. etc.

2. In the domestic fowl we have an experimental animal which has the highest possible susceptibility and in which the experimental infection appears to be invariably fatal.

3. Experimental fowl plague in the fowl is an acute septicæmic disease, the virus existing not only in the cells but also free in the body fluids (serum, pericardial fluid, peritoneal fluid etc.) in which it is present both in a high degree of concentration and also apparently in a homogeneous and stable suspension, as is shown by the regularity of the results of virulence tests made with dilutions of these fluids.

4. The virus is of particular interest in that it behaves quite differently in different species of birds. Thus, while in the fowl it gives rise to an acute septicæmic condition with no marked cerebral symptoms, in the young goose and in the pigeon it definitely attacks the central nervous system. In the young goose the disease is much

less acute than in the fowl, death taking place after from five to nine days with definite cerebral symptoms (paralysis etc.) and at the time of death the virus may be entirely absent from the blood although the brain is highly virulent.

A consideration of these points suggested further experimental investigation of the disease. The particular strain of the virus used for the experiments was from an outbreak of the disease in England reported by Rice (1922).

LOCALIZATION OF THE VIRUS IN THE BLOOD.

In fowls inoculated with fowl plague the blood at the time of death contains the virus in a very high concentration. Maggiora and Valenti (1903) found in one case that 1/31,000,000 c.c. of the virulent blood killed a fowl in 60 hours, and Krumwiede, Gerber and Provost (1925) were able to infect a fowl by the subcutaneous injection of a dose of virulent blood as small as 1/1,000,000,000 c.c.

If the virulent blood is defibrinated and centrifuged and the clear serum and the layer of deposited corpuscles separately tested, the latter are found to be considerably more virulent than the serum. Russ (1906) in a number of experiments found that the corpuscles, even after being washed three times in saline, were generally from ten to a hundred times more virulent than the serum.

By treating the clear virulent serum with washed corpuscles of the normal fowl or guinea-pig and then centrifuging the mixture, the virus was found to have been largely removed from the serum and to be present in a relatively high concentration in the corpuscular layer. From these results he concludes that it is probable that the virus is merely adsorbed by the corpuscles and is not actually contained in them.

Doerr and Pick (1915) made an interesting series of experiments in which they injected the virus into the circulation of naturally resistant animals (rabbits, guinea-pigs and frogs) and observed the length of time for which the virus could be subsequently detected in the blood. The virus was injected in two forms, viz.:—

1. The clear virulent serum of sick fowls.
2. The washed blood-corpuscles of sick fowls.

It was found that in the former case the virus had disappeared from the blood in from one to two hours, whereas when the washed corpuscles were used the blood remained virulent for more than twenty-four hours. These results appear to suggest that the virus is actually located within the corpuscles and in this way is shielded from the action of the serum of the new host.

In their paper Doerr and Pick speak of the corpuscles used as "washed red corpuscles" and it seems to have been tacitly assumed that the cells of the blood with which the virus is associated are the red blood-corpuscles but, so far as the writer is aware, the question of which of the cells of the blood are actually concerned has not been gone into.

In order to investigate this point, a fowl was inoculated with the virus and 24 hours later was bled to death from the jugular vein and the blood oxalated to prevent clotting. The oxalated blood was centrifuged, the supernatant

plasma and buffy coat removed as far as possible, the deposited red cells resuspended in saline and the suspension filtered through absorbent cotton-wool to remove any remaining leucocytes. This process of centrifuging and filtration was carried out six times, after which a portion of the almost solid mass of red corpuscles was taken up in a capillary measuring pipette and a series of suspensions of known concentration made and tested on fowls, the dilutions being made in multiples of ten.

It was found that, while the whole blood killed a fowl at 1/100,000 c.c. and not at 1/1,000,000 c.c., the washed red blood-corpuscles only killed at 1/10,000 c.c. and not at 1/100,000 c.c. Assuming that the red blood-corpuscles make up roughly 50% of the volume of the whole blood, the experiment shows that they contain only about one-twentieth of the amount of the virus present in the whole blood.

A further and more complete test was then made in which the plasma, the white cells (*i. e.* all the cells of the blood other than the red corpuscles), and the red corpuscles were separated from the blood and their degree of virulence separately tested. A fowl which had been inoculated with the virus 25 hours previously was bled to death and clotting prevented by the addition of sodium citrate.

Preparation of the citrated plasma :—This was carried out at once so as to eliminate as far as possible the breaking down of the cells and possible escape of the virus into the plasma. The citrated blood was centrifuged, the clear supernatant plasma pipetted off and again spun in the centrifuge, after which the upper layer of the plasma was removed and centrifuged for a third time in a smaller tube. It was noted that even on the third centrifuging a small but distinct deposit of cells was thrown down.

Preparation of the white cells :—After pipetting off the supernatant plasma, the white cell layer was floated off, emulsified in citrated plasma and spun in the centrifuge, the white cell layer again removed emulsified in plasma and recentrifuged in smaller tubes.

This process was carried out five times yielding a whitish sticky mass of white cells, a known volume of which was taken up with a capillary measuring pipette. The material only entered the pipette with difficulty and when blown out into citrated saline had the form of a thread. It was however easily broken down by shaking with glass beads so as to form the first suspension from which the further dilutions were made.

Citrated plasma was used in this experiment for washing the cells in the hope that it would be less injurious than saline, but no marked difference was observed.

Preparation of the red cells :—The white cell layer having been poured off the deposit in the centrifuge tubes, the red cells remaining were resuspended in saline, the suspension centrifuged and the top layer again discarded. This was done twice and the cells then suspended in warm saline at 37° C. and filtered through absorbent cotton-wool. It was noticed that a certain amount of hæmolysis had taken place although the wool had been previously boiled in saline in order to remove any possible hæmolytic chemical present.

Suitable dilutions of the above suspensions having been made these were tested on fowls by intramuscular injection as shown in Table I.

TABLE I.

Test of Virulence of Citrated Plasma.

Serial number.	Dose.	Result.
219	1/1,000,000 c.c.	Lived.
221 *b*	1/100,000 ,,	Lived.
222 *b*	1/10,000 ,,	Lived.
220	1/10,000 ,,	Died (48 hours).

Test of Virulence of Red Cells.

221	1/100,000,000 c.c.	Lived.
222	1/10,000,000 ,,	Lived.
223	1/1,000,000 ,,	Lived.
224	1/100,000 ,,	Lived.
225	1/10,000 ,,	Died (39 hours).

Test of Virulence of White Cells.

226	1/10,000,000,000 c.c.	Lived.
227	1/1,000,000,000 ,,	Lived.
228	1/100,000,000 ,,	Lived.
229	1/10,000,000 ,,	Lived.
230	1/1,000,000 ,,	Died (42 hours).

The virulence of the plasma, red corpuscles and white cells may, therefore, be summarized as follows: —

	Virulent at—	Not virulent at—
Plasma	1/10,000 c.c.	1/100,000 c.c.
Red corpuscles	1/10,000 ,,	1/100,000 ,,
White cells	1/1,000,000 ,,	1/10,000,000 ,,

Under the conditions of the experiment the concentration of the virus in the white cells appears to be of the order of one hundred times the concentration in the red corpuscles or in the cell-free plasma, but this figure is naturally very approximate as the serial dilutions used in the tests were made only in multiples of ten. Unfortunately, owing to the great differences existing in the virulence of the blood of individual fowls, an exact determination would necessitate the use of a very large number of birds if the whole possible range were to be covered at close intervals.

Obviously the methods used for the separation of the red and the white cells leave much to be desired, particularly as regards the latter which suffer seriously during the process, but the separation of the white cells in any quantity from the blood without considerable damage to their structure does not appear to be easy. When separated from the red cells by centrifuging and washing, the white cells of the fowl rapidly break down so that when suspensions in saline are allowed to stand for any time they are converted into a jelly-like mass. This is not prevented by the presence of oxalate or citrate nor by the avoidance of excessive alkalinity by the use of saline buffered with M/100 phosphate to pH.=6·9; it takes place equally in Ringer's solution and in saline and also in normal fowl's serum inactivated for half an hour at 56°C.

The only practical method of dealing with the difficulty found was to carry out the centrifuging and washing as quickly as possible.

The experiments, however, seem to leave no doubt that the virus of fowl plague in the blood is associated mainly with the white cells, and in view of the ease with which these break down, it appears not improbable that the relatively small amount of the virus found in the cell-free plasma and in association with the red corpuscles may result from the breaking down of the white cells and the consequent liberation of the virus. The term " white cells " is here used as representing all the cells in the virulent blood other than the red corpuscles as no attempt has yet been made to determine the relative virulence of the different types of white cell or of the blood platelets.

An interesting parallel to this location of the virus of fowl plague in the white cell layer is found in the case of cattle plague. As early as 1902, Nicolle and Adil Bey (1902) in their third memoir on cattle plague suggested that the virus of the disease probably existed in the leucocytes of the virulent blood, and Todd and White (1914) were able to show that the concentration of the virus in the white cell layer is approximately one hundred times the concentration in the washed red corpuscles. In virulent cattle plague blood, however, it is possible by continued centrifuging to render the plasma entirely non-virulent, which cannot be done in the case of fowl plague.

Similarly in the blood of human typhus cases Nicolle, Conor and Conseil (1912) found that the virus exists in a high degree of concentration in the washed leucocytes—one cubic millimetre of the cells being sufficient to infect a monkey, while five cubic centimetres of the washed red corpuscles were without effect and the clear centrifuged serum was non-virulent in a dose of one cubic centimetre when injected subcutaneously in a susceptible individual.

In the case of herpes, Zinsser and Tang (1926) made the interesting observation that when the brain of animals infected with herpes was ground with sand and emulsified and centrifuged at high speed for an hour, rabbits inoculated with the almost clear supernatant fluid died often a day or two sooner than those inoculated with the sediment, and these observers suggest that this result may be explained by the breaking down of the cells and the liberation of the intracellular virus.

An examination of the behaviour of other ultramicroscopic viruses in this respect would be of the greatest interest.

ATTEMPTS TO CULTIVATE THE VIRUS.

Marchoux (1908) reported the successful cultivation of the virus in defibrinated normal fowl blood. The cultures were made in test-tubes of 20 mm. diameter containing a column of defibrinated fowl blood overlying a column of glucose peptone agar, and were grown aërobically. Inoculation of the tubes was made by means of a fine platinum wire dipped to a depth of one centimetre into virulent blood and the cultures grown for three days. In this way a series of ten successive passages was made.

Marchoux's work was repeated and confirmed by Landsteiner and Berliner (1913) who carried out nine consecutive passages of the virus in this medium —each passage representing a dilution of 1 in 200. After the nine passages the virulence of the inoculated medium was the same as that of the original

blood. Cultures made in ascitic fluid, fowl serum, rabbit's blood, and fowl's blood laked by freezing were all negative, and the authors conclude that the presence of intact red blood-corpuscles is probably necessary, but that their experiments were not exhaustive enough to settle the question definitely.

A series of attempts was made to repeat this work, using the English strain of the virus and the technique given by Marchoux, but in no case was multiplication of the virus observed. Neither Marchoux nor Landsteiner and Berliner state the temperature at which the cultures were grown, so various temperatures from 37°C. to 42°C. were tried, and both aërobic and anaërobic cultures were made both in whipped and in oxalated blood. In view of the constantly negative results the experiments were discontinued, but it is hoped to make further trials with another strain of the virus.

PASSAGE OF THE VIRUS THROUGH MUCOUS MEMBRANE.

It has been recorded by numerous observers that birds are easily infected by means of virulent blood or secretions dropped into the conjunctival sac, mouth, or nasal aperture. In order to verify this, a number of fowls were infected with various virulent materials, but, to avoid the possibility of infection by swallowing, the cloaca was used as the site of infection. The results of the infection of 14 fowls in this way are given in Table II.

TABLE II.

Fowls Infected by the Cloaca.

Material used for infection.	Number of fowls.	Result.	
		Infected.	Negative.
Virulent blood	4	3	1
Virulent citrated plasma	1	0	1
White cell layer (vir. blood)	1	1	0
Mouth secretion	3	2	1
Cloacal secretion	5	5	0

In the earlier experiments the cloaca was swabbed with a cotton-wool swab moistened with the virulent material, but it was found—at any rate in the case of infection with the cloacal secretion—that swabbing was unnecessary and that if the cloaca of a fowl dead of the disease was swabbed with cotton-wool, and the swab used to infect a few cubic centimetres of saline, infection of a healthy bird could be effected by allowing one or two drops of the infected saline to fall from a pipette into the everted lips of the cloaca, the mucous membrane not being touched by anything except the infective fluid.

It thus appears that the penetration of the mucous membranes by the virus takes place with ease in the complete absence of any mechanical injury. This will be again referred to in connection with the natural spread of the disease.

METHOD BY WHICH THE DISEASE IS SPREAD UNDER NATURAL CONDITIONS.

In spite of the large amount of attention which has been devoted to fowl plague, both in Europe and in the United States, the means by which the spread of the disease takes place under natural conditions is at present quite unknown.

The severe outbreaks which have been repeatedly traced to the introduction of infected birds into previously healthy districts show that the diseased fowl itself is undoubtedly the most important means of disseminating infection, but as regards the mechanism by which this is brought about we are in complete ignorance.

In view of the intense virulence of the nasal, buccal, and cloacal secretions of infected fowls it would naturally be inferred that such birds would be highly infective by contact to healthy fowls. Curiously enough, when tested experimentally, this is not found to be the case, and while certain observers have reported successful results from simple contact, the vast majority of workers have been unable to infect in this way. Thus, Doerr and Pick (1915), Marchoux (1910), Doerr and Zansky (1924), Krumwiede (1925), Beaudette (1925), Miessner (1926), all found that healthy fowls placed in the same cage with infected birds did not as a rule contract the disease. A striking experiment was carried out by Doerr and Pick (1915), in which a fowl that had died of the disease was taken and washed with benzine to kill any ectoparasites, the thorax and abdomen were then laid open and the dead bird placed in a cage with a healthy fowl. The latter pecked the viscera of the dead bird and ate portions of the liver but did not contract the disease.

An excellent summary of previous work on the direct infectivity of fowl plague is given by Doerr and Zdansky (1924).

Direct infection can, however, undoubtedly occur and Krumwiede (1925), has shown that if a chicken dead of the disease is taken and his bill sharply tapped against the skin of a healthy fowl, this may be sufficient to produce the disease. Still, it is remarkable how rarely contact infections are observed under laboratory conditions. On the other hand, the infectivity of the disease under special conditions is shown by the case reported by Mohler (1926), where, in a consignment of 3300 birds in one railway car, 3000 died in five or six days.

Quite recently Doyle (1927) has confirmed the fact that a healthy fowl does not contract fowl plague when put in a cage with an infected bird, but he finds that the disease invariably results if the amount of infection is increased by the employment of three or four infected birds.

The possibility that the virus may be transmitted by some ectoparasite of the fowl has been considered and Hertel (1904) was able to infect a bird by the injection of the crushed bodies of 24 lice taken from a fowl dead of the disease. In this case, however, the infection may have been due to direct transfer of the infective blood contained in the lice, as Centanni (1902) carried out a certain number of experiments both with living and dead lice (Dermanyssus avium) but was unable to transmit the disease in this way. Marchoux (1910) also obtained negative results with Argas persicus.

It is doubtful if the possible *rôle* of ectoparasites in the transmission of fowl plague has yet been adequately investigated but the general experience that a healthy bird, placed in the same cage with a sick fowl which is allowed to die in the cage, does not as a rule contract the disease has naturally tended to discourage work along these lines.

In reviewing the various factors commonly regarded as responsible for the natural spread of the disease—such as oral infection through contamination

of food and drink, injuries to the skin, etc.—it is somewhat remarkable that the possibility that fowl plague is spread by sexual intercourse does not appear to have been suggested. The knowledge of the intense infectivity of the cloacal secretion in infected birds and the fact that merely bringing a drop of this secretion into contact with the uninjured cloacal mucous membrane of a healthy fowl is sufficient to cause infection, renders it more than probable that sexual intercourse between an infective and a healthy bird would result in the infection of the latter.

In order to investigate this point a certain number of experiments were carried out by placing a healthy bird in the same cage with an infected fowl of the opposite sex. A total of eleven experiments were made, in four of which the infected bird was a cock and in seven a hen. In three cases infection took place. In the first of these a hen which had been infected with fowl plague 24 hours previously and a healthy cock were placed in a clean cage, and three hours later the sick hen was removed. The cock sickened and died 48 hours after being placed in contact with the sick bird, and 45 hours after the latter was removed from the cage. In this case the time of contact was only three hours and the cage was a clean one.

In both the second and third experiments where infection occurred the two birds were placed in a cage and then one of them (in each case the cock) infected with the virus. As soon as the infected bird died it was removed and its companion left in the cage.

In these two experiments the hens died in 2–2½ days and 3–4 days respectively, but in the case of the last bird death took place during the week-end so that the exact time was not observed.

The proportion of three infections out of eleven experiments appears at first sight somewhat small, but under the artificial conditions of the experiment a number of factors come into play which render infection less probable than under natural conditions. Thus, apart from the fact that the majority of the tests were made in late autumn and winter—a time at which the sexual activities of the fowl are greatly repressed—the experimental disease is most acute, death usually taking place in some 44 hours, so that the period during which the fowl is infective is comparatively short and it is impossible to ensure that sexual connection has taken place during this time, particularly as the birds are caged and under quite artificial conditions. In a naturally occurring outbreak, on the other hand, the disease is as a rule much less acute—killing in from three to eight days—and when the birds are under their natural conditions the probability of sexual intercourse taking place during the infective period must be very much greater.

CONCLUSIONS.

1. The virus of fowl plague presents special advantages for the study of the ultramicroscopic viruses.

2. In the centrifuged blood of infected fowls the virus is found mainly in the white cell layer, the concentration of the virus in this layer being roughly one hundred times that in the clear plasma or in the washed red blood-corpuscles.

In this respect fowl plague presents interesting analogies to cattle plague and to human typhus.

3. Attempts to repeat Marchoux's and Landsteiner and Berliner's work on the cultivation of the virus *in vitro* gave entirely negative results with the particular strain of the virus used.

4. The cloacal secretions of sick birds are very highly infective and the infection of healthy fowls is produced with ease by dropping a dilution of these secretions into the cloaca without otherwise touching the mucous membrane.

5. Although the possible *rôle* of ectoparasites of the fowl in the natural transmission of the disease has not yet been adequately investigated, there is reason to believe that direct infection may be commonly effected by means of the sexual intercourse between sick and healthy birds.

I am indebted to Mr. F. C. Minett, B.Sc., M.R.C.V.S., of the Ministry of Agriculture for the English strain of the virus, to Prof. Miessner for his strain of the goose virus and to Mr. F. M. Doyle, F.R.C.V.S., for the strains isolated by de Bliech and Staub.

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A NOTE ON THE CULTIVATION OF THE VIRUS OF FOWL-POX.

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MANY attempts have been made to cultivate ultra-microscopic viruses on artificial media, but except in the case of the virus of bovine pleuro-pneumonia first cultivated by Nocard and Roux (1901) in fluid media and later by Bordet (1910) on solid media these attempts have for the most part ended in failure.

More successful efforts have, however, been made to cultivate ultra-microscopic viruses by employing the technique of tissue culture, it having been noted by Levaditi (1913) that the virus of anterior poliomyelitis retained its activity in tissues surviving outside the body.

Steinhard and Lambert (1914) found that vaccinia virus proliferated when guinea-pig cornea which had been immersed in a suspension of the virus was grown "in vitro," while later Harde (1915), Parker and Nye (1925) and Craciun and Oppenheimer (1926) also successfully used tissue culture methods for the growth of vaccinia virus.

Efforts to cultivate the virus of fowl-pox have not been numerous. Bordet and Fally (1910) believed that they had grown the organism of fowl diphtheria on a medium similar to that used for the cultivation of the bacillus of whooping cough. Their observations have not however been confirmed.

In the present experiments a number of artificial media were tried including broth, fowl serum broth, Hartley's broth with and without the addition of fowl serum and chick embryo, defibrinated chicken blood and Bordet's potato glycerin blood agar. No evidence was obtained of any multiplication of the virus in any of these media. Survival of the virus in artificial media was in no instance longer than in Locke's solution at a pH = 7·2, nor was there any evidence that the virus had been inactivated by the disappearance of a specific chemical factor necessary for its infectivity.

The method finally used for the cultivation of the virus of fowl-pox closely followed that described by Carrel and Rivers (1927) for the production of quantities of vaccinia virus "in vitro."

Material from ten day old fowl-pox lesions was ground in a mortar with sand and Locke's solution and lightly centrifuged to throw down gross particles. The mixture was then passed through a Berkefeld filter. Dilutions of the filtrate 1 in 10, 1 in 100, 1 in 1,000 1 in 10,000 and 1 in 100,000 were prepared and their activity tested on the skin of chickens. The filtrate was found to be active in dilutions as high as 1 in 10,000. 1 c.c. of the 1 in 1,000

dilution of the filtrate was then mixed with the skin and brains of an embryo chicken, 12 to 15 days old, and allowed to stand in the ice chest for 48 hours. Equal amounts of the embryo and virus suspension were then distributed in small flasks each containing 5 c.c. of chicken plasma, dilutions of one of the flasks being tested on chickens. The flasks were covered with rubber caps to prevent evaporation and were incubated at 37° C. for 4 days. At the end of this time embryo and coagulum were removed from the flasks, ground in a mortar with sterile sand and Locke's solution was added to form dilutions from 1 in 10 to 1 in 100,000. The infectivity of these dilutions was again tested on chickens. 1 c.c. of the first culture, diluted 1 in 1,000 was added to a fresh lot of embryo pulp and incubated for four days, similar subcultures being continued to the fourth generation. The infectivity of dilutions of each culture was tested on chickens with the results shown in the Table.

	Dilutions of culture.				
	1/10	1/100	1/1,000	1/10,000	1/100,000
1st culture before incubation	+	−	−	−	−
1st „ after incubation	+	+	+	−	−
2nd „ „ „	+	+	+	+	−
3rd „ „ „	+	+	+	+	−
4th „ „ „	+	+	+	−	−

In control flasks containing only virus and chicken plasma, the virus failed to survive for four days. In another series of flasks chicken embryo was replaced by mouse or rat embryo skin and brain. Here again the fowl-pox virus did not survive for four days. There is thus evidence that the virus of fowl-pox requires for its growth the presence of active ectodermal cells from the fowl.

CONCLUSION.

A method is described for cultivating "in vitro" the virus of fowl-pox.

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SERO-DIAGNOSIS OF SYPHILIS BY MEINICKE'S MACRO-REACTION AND MICROREACTION.

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THE attractiveness of Meinicke's reactions for the sero-diagnosis of syphilis is due to their simplicity, the speed in obtaining a result, the relatively small amount of serum required and the ease with which a definite result can usually be obtained.

It is admitted that no flocculation or precipitation reaction up to date can compare in reliability with the Wassermann reaction, and that the latter is invariably the court of appeal in doubtful cases. Meinicke's reactions have a great reputation on the Continent for reliability, and it was with a view to corroborating these findings that the following comparative tests were undertaken.

The test is best performed with Meinicke's own extract, which may be purchased from the Adler Apotheke, Hagen, Westphalen. The efficacy of the antigen depends mainly on the properties of the balsam of Tolu used in preparation. We were fortunate in obtaining a good sample of balsam, and succeeded in getting consistent and good results with a reagent prepared by ourselves which approximated in colour and consistency with that purchased from the Westphalen works. Preliminary practice in reading the reaction was first obtained with the purchased reagent.

Principle of the test.—When a given quantity of a syphilitic serum is mixed with a given quantity of the suitably diluted reagent, a turbidity occurs which is visible at the end of one hour and maximum in two hours. With strongly positive sera there eventually occurs complete precipitation. No turbidity and no precipitation occur with a definitely negative serum, and the entire reaction in a positive serum can be inhibited by the addition of one drop of 40% formaldehyde.

The latter phenomenon gives a method of controlling the reaction, in that any turbidity due to hæmolysis or cloudiness in the specimen can be allowed for. In the microreaction the principle is the same. The serum to be tested is put up as a hanging-drop preparation with the suitably diluted reagent and very definite agglutination of particles may be observed at the end of one hour.

Practice of the Test.

Full details of the original method and the various refinements may be found in Meinicke's publications (1924), but the practice as carried out by us may be briefly summarized here.

Macroreaction.—The sera to be tested are centrifuged until no corpuscles are present and distributed without previous heating into 5″ × ¼″ tubes. All glassware must be perfectly clean and dry. Two tubes are required for each test. 0·2 c.c. of clear serum is added to each tube, and to one of them (the control tube) one drop of 40% formaldehyde. 1 c.c. of diluted balsamized heart extract is required for each tube, and it is preferable not to prepare this in batches of more than 10 c.c.

In preparing the reagent for testing five sera with controls, place into one dry tube 0·25 c.c. of balsamized heart extract, and into another tube 10 c.c. of a solution containing 3% pure sodium chloride and 1·5% pure ammonium chloride. Put both these tubes in the water-bath at 40° C. for 10 minutes. At the end of this time rapidly mix the contents of the two tubes, allow to stand until the correct turbidity has developed and then distribute 1 c.c. to each of the serum tubes, including the controls. The operation of distribution should not occupy more than a minute. Shake the tubes and allow to stand at room temperature. Read the results at the end of 1 hour, 2 hours, and 24 hours.

Microreaction (Meinicke, 1925).—In this reaction a control is made with a known negative serum. A series of clean, dry, fat-free coverslips are carefully ringed with vaseline and a series of well-slides placed ready for their reception. The balsamized heart extract and diluting fluid are warmed as for the macroreaction, but the proportion of extract to diluting fluid is 1 in 25 instead of 1 in 40. A different strength of extract is required. The reagents are mixed and, when the correct turbidity has developed, one large loopful of the mixture is transferred to each coverslip. Using a loop one-sixth the size of the previous one, a loopful of each serum to be tested is quickly mixed with the reagent on the coverslip. The hanging-drop preparation is mounted and examined with the ⅔″ and ⅙″ lenses at the end of 1 hour and then 2 hours.

The main difficulty of the reaction is the tendency of the diluted extract to auto-agglutinate. For this reason it is preferable not to perform more than ten tests with one lot of diluted extract, and for the last preparation of each batch of ten to be made with a known negative serum.

Composition of the Reagents.

Macroreaction.—The balsamized heart extract as used by us was made by adding 1½ parts of 5% alcoholic solution of balsam of Tolu to 4 parts of alcoholic extract of human heart prepared as described by Fildes and McIntosh. These proportions and the required amount of diluting fluid were found by titration with known positive and negative sera. It will be noted that the extract is diluted 1 part in 40 with the sodium and ammonium chloride solution.

Microreaction.—The balsamized heart extract was made by adding 1 part of 5% alcoholic solution of balsam of Tolu to 4 parts of alcoholic extract of human heart. This is diluted 1 part in 25 with the sodium ammonium chloride solution.

Difficulties of the Test.

In doubtful cases considerable practice is required in reading the macro-reaction before a reliable opinion can be given. Definite positive sera give a turbidity which can be easily demonstrated if the tubes are held about two or three yards from the cross-bar of a window. In positive cases the image of the cross-bar is either occluded or obscured. It is preferable to read the result in daylight, and in doubtful cases it will be found that the 24-hour precipitation reaction is more reliable than a questionable turbidity.

The microreaction is definitely more sensitive, and is of small value in doubtful cases.

The question as to what constitutes the correct turbidity of the mixed reagents is again a fact which requires experience. It is obviously a point which must be decided by a particular worker for his particular reagents, though its determination requires only a small experience. Mixtures which are too turbid give unreliable results, and mixtures which are too clear give a turbidity which it is difficult to estimate. The correct turbidity should be determined by titration with known positive and negative sera. The main difficulties have always been found with treated cases. Quantitative estimations are impossible to perform, and it is better to give the result as either positive or negative without any estimate as to the degree of reaction.

Results.

Results as compared with Wassermann reaction (method of Fildes and McIntosh) were as follows:

Using own reagents.—254 sera were examined by macro-reaction, and 235 by micro-reaction.

By Macro-reaction.

Positive	Meincke	and	positive	Wassermann	reaction	78	agreement 91%.
Negative	,,	,,	negative	,,	,,	153	
Positive	,,	,,	,,	,,	,,	11	disagreement 9%.
Negative	,,	,,	positive	,,	,,	12	
						254	

By Micro-reaction.

Positive	Meinicke	and	positive	Wassermann	reaction	57	agreement 88·5%.
Negative	,,	,,	negative	,,	,,	151	
Positive	,,	,,	,,	,,	,,	12	disagreement 11·5%.
Negative	,,	,,	positive	,,	,,	15	
						235	

In analyzing the cases which were not in agreement with the Wassermann reaction it was found that they were invariably cases which were undergoing, or had undergone, treatment. No difficulties were experienced in a straight diagnostic test.

Using Meinicke's reagents.—In the preliminary practice of the reaction, before embarking upon the preparation of a synthetic extract we made use of Meinicke's own reagents bought from Westphalen. In examining 160 sera we found that 90% were in agreement with the Wassermann reaction in the macro-reaction and 93% in the micro-reaction—figures which are in quite close agreement with those recorded above. It is noteworthy that Meinicke's heart extract is an alcoholic extract of a previously ether-extracted heart to which has been added alcoholic balsam of Tolu. In our case the heart extract was merely alcoholic, made without previous ether extraction.

CONCLUSIONS AND COMPARISONS.

In working with this reaction we have found it quite definitely reliable in untreated cases, and it is therefore of distinct value for diagnostic purposes. The simplicity and quickness of performance are points in its favour.

The micro-reaction is of particular value in children with congenital syphilis. Continental findings indicate that the reaction is more sensitive than the Wassermann reaction. Thus Kovacs (1926), in investigating 520 cases by the micro-reaction, found an agreement with the Wassermann reaction in 88·7% of cases. Of the deficiencies 9·2% gave a positive Meinicke and negative Wassermann reaction, whilst 2·1% gave a positive Wassermann and negative Meinicke reaction. In our experience the discrepancies as compared with the Wassermann reaction have never indicated that Meinicke's reaction is the more sensitive. In our series of cases the results have been compared with the identical Wassermann method employed for confirming the sensitivity of the sigma reaction. The latter reaction was found by Dreyer and others, in testing 894 cases, to be more sensitive than the Wassermann test for treated cases, but to give consistent agreement in straight diagnostic tests. From this we conclude that the sigma reaction is more sensitive than Meinicke's reaction.

Kahn (1925), comparing his reaction with the Wassermann reaction in 58,170 cases, claims the high percentage agreement of 94·64 in both treated and untreated cases. In our opinion Meinicke's reactions are distinctly less trouble to perform and more easy to read than either the Kahn or sigma reactions. The value of Meinicke's reactions is mainly that of orientation, the progress of treatment being controlled by the Wassermann test with or without Meinicke's reactions as confirmatory evidence.

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VACCINATION OF GUINEA-PIGS AGAINST TUBERCULOSIS WITH B.C.G.

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THIS communication deals with four experiments which have been carried out during the last three years in an endeavour to repeat Prof. Calmette's work on the prophylaxis of tuberculosis in guinea-pigs with vaccine B.C.G. In each experiment a group of animals received one or more injections of the vaccine subcutaneously or intravenously; after intervals of from 5 to 12 weeks to allow for the development of immunity, the vaccinated guinea-pigs and an approximately equal number of controls of similar weights were given an infecting dose of virulent *B. tuberculosis*. All the animals were observed regularly and weighed at weekly or fortnightly intervals. Post-mortem examinations were performed on all dead guinea-pigs. Those animals which died from intercurrent diseases too early in the experiment to develop tubercular lesions visible at autopsy as a result of the infecting dose were excluded from the comparison of the protected and unprotected groups.

Experiment 1.—This experiment is to be regarded as a preliminary one only (Chart 1). Five guinea-pigs, which had received 20 mgm. of B.C.G. subcutaneously 12 weeks previously, were given an infecting dose of virulent organisms (human strain) contained in a suspension of triturated gland from a tubercular guinea-pig. Six guinea-pigs were injected with gland only as controls, and were all dead from generalized tuberculosis at the end of 16th week, the average period of survival being 11 weeks. When the last of the control guinea-pigs died, all the vaccinated guinea-pigs, apparently fit and well-nourished, were killed; at autopsy they all showed evidence of generalized tuberculosis, though the lesions they exhibited were less advanced than in the control animals which had died at an earlier date as a result of their infection.

In later experiments great care was taken to select guinea-pigs of exactly comparable weight and physical condition for the two groups, but in the preliminary experiment the vaccinated animals were in rather better condition and slightly heavier than the controls at the time of infection with virulent organisms. Even when this was taken into consideration the results were of such promise that further experiments were arranged.

Experiment 2.—In this experiment the vaccinated guinea-pigs were given the same dose of B.C.G. (20 mgm.) as in the previous experiment, but the infecting dose was 0·5 mgm. of the virulent human strain we use for the routine infection of guinea-pigs for the standardization of tuberculin. The interval between the injection of the living vaccine and the injection of

virulent culture was 11 weeks. The period of survival of 10 vaccinated and 15 control guinea-pigs is represented in Chart 2. There were 3 survivors in the immunized group, which were killed in the 20th week and were found to be well infected with tuberculosis. If these animals had died from tuberculosis in the 20th week the average period of survival of the vaccinated animals would have been 17 weeks, or 4 weeks longer than that of the controls.

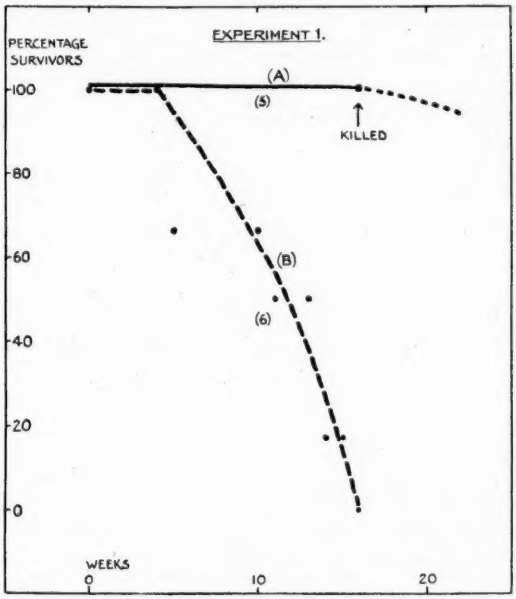

CHART 1.

EXPERIMENT 1.

Figures in brackets indicate number of guinea-pigs under experiment in each group.

A. *Vaccinated guinea-pigs* (————————).
Injected subcutaneously with 20 mgm. B.C.G. After 12 weeks injected subcutaneously with 0·25 c.c. suspension of gland from guinea-pig infected with virulent human strain of *Bacillus tuberculosis*.
Average period of survival, over 16 weeks.

B. *Control guinea-pigs* (— — — — — — — —).
Injected with guinea-pig gland only.
Average period of survival, 11 weeks.

Experiment 3.—An attempt was made in this experiment to obtain a greater difference in the period of survival of the two groups by giving five injections of 20 mgm. each of B.C.G. subcutaneously, instead of one only as in previous experiments, and by reducing the infecting dose of virulent culture to 0·25 mgm. The difference between the vaccinated and unvaccinated

guinea-pigs in this experiment did not, however, prove any greater than in similar groups in Experiment 2.

The interval between the last injection of B.C.G. and the injection of virulent culture was 5 weeks. Twenty-one guinea-pigs in the vaccinated group lived on an average just under 7 weeks, or approximately 2 weeks longer than 25 controls (Chart 3).

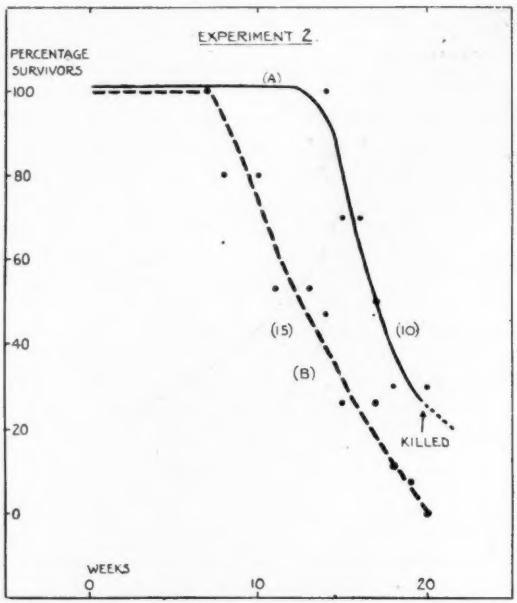

CHART 2.

EXPERIMENT 2.

Figures in brackets indicate number of guinea-pigs under experiment in each group.

A. *Vaccinated guinea-pigs* (————————).
Injected subcutaneously with 20 mgm. B.C.G. After 11 weeks injected subcutaneously with 0·5 mgm. of a virulent human strain of *Bacillus tuberculosis*.
Average period of survival, over 17 weeks.

B. *Control guinea-pigs* (— — — — — — — —).
Injected with virulent culture only.
Average period of survival, 13 weeks.

The average period of survival of a small group of 13 guinea-pigs which had received multiple injections of Koch's bacillary emulsion (B.E.) was only one week longer than that of the controls. One cannot draw conclusions from an experiment on so small a number of animals, but the result is suggestive, and lends support to the observations of other workers on the immunity produced by B.E., dead tubercle bacilli and many allied vaccines.

Experiment 4.—This experiment was arranged as far as possible on the lines recommended by Prof. Calmette, and, by choosing a critical infecting

dose, we hoped to exaggerate the differences between the control and vaccinated guinea-pigs which were evident in the previous experiments. A single injection of the vaccine B.C.G. was given intravenously, followed after an interval of 5 weeks by 0·01 mgm. of the virulent bovine strain used by Prof. Calmette

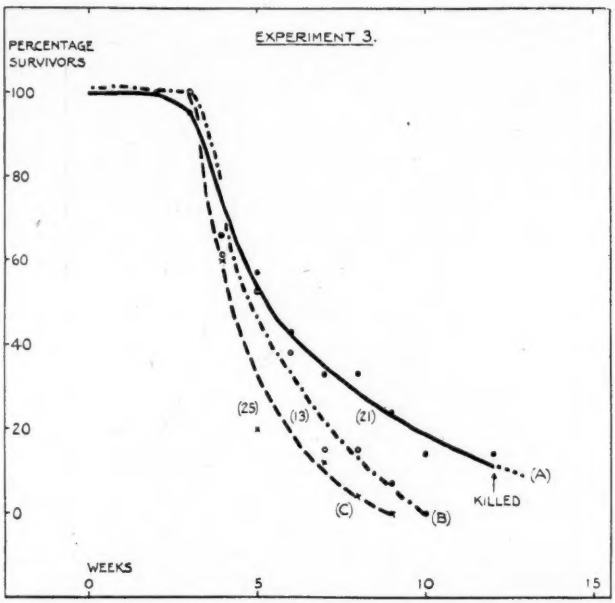

CHART 3.

EXPERIMENT 3.

Figures in brackets indicate number of guinea-pigs under experiment in each group.

Vaccinated guinea-pigs.

(A) (——————). Received subcutaneously 5 injections of 20 mgm. each of B.C.G. in 9 weeks. After 5 weeks injected subcutaneously with 0·25 mgm. of a virulent human strain of *Bacillus tuberculosis.*

Average period of survival, 7 weeks.

(B) (— · — · — · —). Received subcutaneously 5 injections of 20 mgm. each of B.E. in 9 weeks. After 5 weeks injected subcutaneously with 0·25 mgm. of a virulent human strain of *Bacillus tuberculosis.*

Average period of survival, 6 weeks.

Control guinea-pigs (— — — — — — —).

(C) Injected with virulent culture only.

Average period of survival, 5 weeks.

in similar experiments. A 3 weeks' growth of the virulent culture from potato medium was ground in sterile 0·9% saline solution in an agate mortar, and standardized to contain 1 mgm. of tubercle bacilli per c.c. The unfiltered suspension, which was homogeneous to the naked eye and showed only isolated bacilli or small clumps of acid-fast organisms microscopically, was thoroughly

shaken before being used. The infecting dose was 0·01 c.c. (containing 0·01 mgm.), which was placed on the eye-ball with the aid of Trevan's micrometer syringe. Using this syringe and such small amounts, the risk of loss from overflowing on to the cheek would appear to be a negligible one,

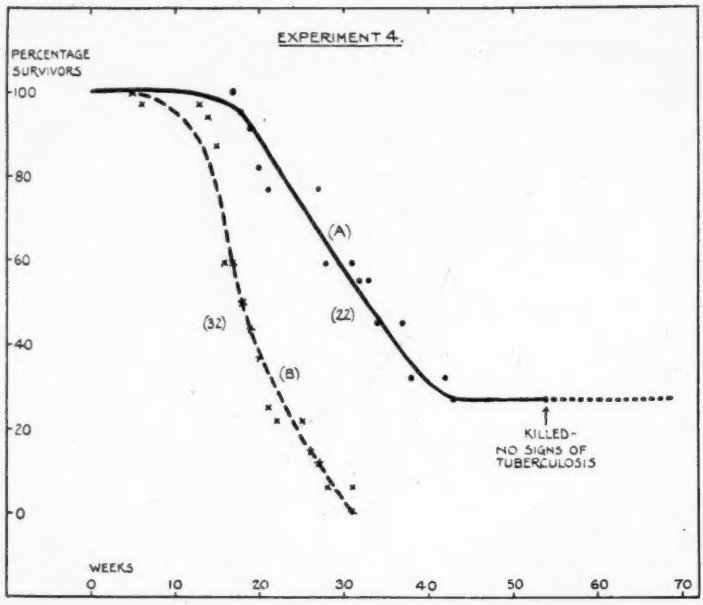

CHART 4.

EXPERIMENT 4.

Figures in brackets indicate number of guinea-pigs under experiment in each group.

A. *Vaccinated guinea-pigs* (————————).
Injected intravenously with 20 mgm. B.C.G. After 5 weeks 0·01 mgm. of a virulent bovine strain (Calmette) of *Bacillus tuberculosis* placed on the eye-ball.
Average period of survival, over 35 weeks, or, excluding 6 tubercle-free survivors, 29 weeks.

B. *Control guinea-pigs* (— — — — — — — —).
Injected with virulent culture only.
Average period of survival, 20 weeks.

and although only a small drop was instilled, many tubercle bacilli must have come in contact with each animal's conjunctiva. In a preliminary experiment 0·01 mgm., of virulent culture placed on the eye-ball infected 9 of 10 guinea-pigs (the tenth animal lived for 10 months and post-mortem showed no signs of infection); 0·0001 mgm., the dose recommended by Prof. Calmette, produced only a few lesions in the lungs of 1 of 10 guinea-pigs. It was

therefore decided that 0·01 mgm. would probably represent a satisfactory "minimal infecting dose" for Experiment 4.

The average period of survival of 32 control guinea-pigs in this experiment (Chart 4) was 20 weeks. In the vaccinated group of 22 guinea-pigs, 6 showed no signs of tuberculosis when killed in the 54th week. When this result is taken in conjunction with the much longer period of survival of the majority of guinea-pigs in the control group, it seems evident that vaccinated animals may be completely protected from infection. Even if these 6 animals are omitted, the vaccinated guinea-pigs lived on an average 29 weeks, or 9 weeks longer than the controls.

These results are to be attributed partly to the very small dose of virulent culture selected to produce tuberculosis, and partly to the route of infection. Calmette has stated that ocular instillation is to be preferred to other methods, because it more nearly approaches natural infection, and does not produce a permanent tubercular focus such as develops after the subcutaneous injection of virulent organisms.

TABLE I.—*Experiment 4: Average Increase in Weight after Infection with Virulent T.B.*

	Average weight when infected.	Average increase in weight—0–10 weeks.	Average increase in weight—0–15 weeks.
Vaccinated B.C.G.:			
22 guinea-pigs	360 gm.	210 gm.	180 gm.
Controls:			
32 guinea-pigs	345 „	140 „	68 „

Table I, showing the average increase in weight of the two groups of guinea-pigs in Experiment 4, lends support to the conclusion that B.C.G. has a restraining influence on the development of tuberculosis in the guinea-pig.

The results obtained in Experiment 4, where a critical infecting dose has been used, are very striking, and numerical analysis indicates that they are significantly in favour of a definite, though by no means complete, immunity to tuberculosis.

In Experiments 2, 3 and 4 there has been no difference in the post-mortem appearances of guinea-pigs belonging to either the vaccinated or the control groups which have died from tuberculosis. Enlargement of the cervical glands was apparent at autopsy in Experiment 4, in which the guinea-pigs were infected by the ocular route.

B.C.G. Does Not Cause Tuberculosis in Guinea-Pigs.

B.C.G. has never seemed to produce any of the lesions of tuberculosis during the period of observation of these experiments. In each experiment 6 or more vaccinated guinea-pigs which have not received an injection of virulent organisms subsequently have remained well for several months, and have shown no signs of tubercular infection when killed. It should be pointed out, however, that Petroff, Branch and Steenken (1927) state they were able to obtain virulent "variants" from cultures of B.C.G.

One of the guinea-pigs in the vaccinated group in Experiment 4 which died shortly before it was due to receive an infecting dose of virulent organisms showed at the autopsy several pulmonary, hepatic and splenic lesions which resembled tubercular granulomata, but were non-infective for 4 other guinea-pigs. A few acid-fast bacilli were seen after prolonged search of a smear prepared from the spleen, and an organism of the Salmonella group was obtained on culture from the same organ. Calmette (1927) has stated that similar lesions may sometimes be seen 3 to 5 weeks after the intravenous injection of large doses of B.C.G. into rabbits and guinea-pigs, and are to be ascribed to emboli of the capillaries and to the formation of giant-cells around the clumps of bacilli. They never progress to calcification, and disappear spontaneously after some weeks.

It may be added in passing that B.C.G. renders the majority of guinea-pigs slightly but definitely sensitive to tuberculin, injected intracutaneously.

Effect of B.C.G. on General Health of Guinea-pigs.

In Experiments 1 and 2 a single subcutaneous injection of B.C.G. produced small abscesses locally in the majority of guinea-pigs. In these experiments, and again in Experiment 4, in which the vaccine was given intravenously, the death-rate of the vaccinated animals over a period of several weeks following the injection was not appreciably higher than of the uninoculated controls.

In Experiment 3, in which five injections of 20 mgm. each of B.C.G. were given subcutaneously, the injections were given at first at weekly intervals but were not tolerated very well; the animals developed extensive abscess-formation at the sites of injection, and many of them died of intercurrent diseases. Towards the end of the course the interval between injections was increased to 3 weeks, but when the infecting dose of virulent culture was given 14 weeks after the first injection or 5 weeks after the last, 25 of the 50 guinea-pigs originally included in the group had died, representing a 50% mortality. Another 4 animals died of a Salmonella infection a few days later. In this group of animals, therefore, 58% of deaths were not attributable to tubercular infection and have been excluded from Chart 3. The mortality in the control group in the same period was 37%.

In all four experiments deaths not attributable to tuberculosis are not included in the charts, but, in order to give the full experimental results, are recorded in Table II.

TABLE II.—*Deaths* not *attributable to Tuberculous Infection and excluded from the Charts.*

Number of experiment.	Original number of guinea-pigs.		Dose of B.C.G. in mgms.	From date of *first* injection of B.C.G. to time of injection with virulent T.B.		After virulent T.B. injected.	Deaths not attributable to tuberculosis.	
	Vaccinated with B.C.G.	Controls.		Weeks.	Deaths.	Deaths.	Total number.	Percentage.
1	6	...	20	12	0	1	1	16
	...	6	0	12	0	0	0	0
2	20	...	20	11	8	2	10	50
	...	26	0	11	9	2	11	42
3	50	...	100*	14	25	4	29	58†
	...	40	0	14	12	3	15	37
4	35	...	20	5	8	5	13	37
	...	45	0	5	7	6	13	28

* Five successive doses of 20 mgm. in 9 weeks.

† In a similar experiment with Koch's bacillary emulsion (B.E.), 57% of deaths were not attributable to tuberculous infection.

Statistical Note.

We are indebted to Dr. J. W. Trevan for the following statistical note:

Experiment 1.—The mean time of survival for the vaccinated guinea-pigs was obtained by taking the time at which the five were killed as the survival time for those five. Tested by the second method given by Fisher ('Statistical Methods for Research Workers,' p. 109), the value of t obtained is 2·08, which corresponds to a value of $P < 0{\cdot}1$ and $> 0{\cdot}05$. The mean times of survival are therefore not significantly greater for the vaccinated guinea-gigs; but if the vaccinated guinea-pigs had all lived for 18 weeks, as they probably would have done, the result would have been significant.

Experiment 2.—The value of t obtained in the same way is 3·08 ($P < 0{\cdot}01$), which indicates that there is a high degree of probability that the difference in survival time is statistically significant.

Experiment 3.—For B.C.G. injected guinea-pigs and controls $t = 2{\cdot}22$ ($P < 0{\cdot}05 > 0{\cdot}02$); the difference of survival time of the vaccinated and of the control animals is therefore significant. For B.E. injected guinea-pigs and controls $t = 1{\cdot}25$ ($P = 0{\cdot}2$); the difference is therefore not significant.

Experiment 4.—Of the guinea-pigs in this experiment 6 of the vaccinated were found free from tuberculosis when killed. None of the other controls failed to show tuberculous lesions. For an event which shows 6 successes out of 22 trials the standard deviation is $\sqrt{\frac{6}{22} \times \frac{16}{22}} = 2{\cdot}09$, and the chance against none dying in 32 similar animals injected at the same time is therefore about 300 to 1. If we also use as controls the previous group of 10 unvaccinated guinea-pigs of which 1 failed to become infected the difference is still significant. There is therefore statistically significant evidence that B.C.G. vaccinated guinea-pigs may be completely protected from infection.

The remaining 16 vaccinated guinea-pigs had an average survival time of 28·6 weeks; that of the controls was 19·7 weeks. The value of t is 4·27 ($P < 0{\cdot}01$); the mean survival time of the vaccinated guinea-pigs is significantly greater than that of the controls.

DISCUSSION.

From these experiments it seems clear that by means of B.C.G. guinea-pigs can be partially and in some cases completely protected from infection with a dose of virulent bacilli. The results seem to be worth recording in detail, because there are so many conflicting views in the literature as to the efficacy of any form of tuberculosis vaccine. We believe that the evidence recorded here proves beyond all reasonable doubt that a vaccine which in our hands at least was apparently harmless has given rise to a significant immunity.

From the time of Koch's original experiment showing the modified reaction of an animal to a second dose of virulent bacilli, much evidence has been produced that cultures, attenuated or having a low virulence to the particular animal under test, are able to produce some degree of immunity (*e.g.* Krause, 1926). Frequently the evidence in favour of these experiments has been submitted to much destructive criticism, as in the case of Von Behring's bovo-vaccine.

One has, of course, no guarantee that B.C.G. represents a permanently avirulent strain. Indeed, the recent work of Petroff and his colleagues (1927) suggests that it is capable of dangerous reversion. In our experiments, at least, it has never infected guinea-pigs, and therefore represents a move in the right direction as compared with the partially virulent strains with which most of the sound work of the past has been done.

It is difficult to judge from the literature whether the effect demonstrated here in the case of B.C.G. could be paralleled by vaccines of dead tubercle bacilli, either intact or ground up in the form of B.E., or even by old

tuberculins. Many of these preparations were stated to have been able to prolong the life of guinea-pigs.

Zinsser, Ward and Jennings (1925), using dead tubercle bacilli as a vaccine, kept treated guinea-pigs alive for much longer periods than the controls, although apparently they did not arrange an experiment with an infecting dose sufficiently "critical" to get complete protection.

It seems quite definite that in experiments with guinea-pigs such as we have described, some measurable protection is conferred on the vaccinated animals. Calmette describes a much higher degree of protection in cattle and apes, but as our experiments did not relate to these animals such considerations were outside the scope of the present paper.

SUMMARY AND CONCLUSIONS.

(1) A living vaccine of Calmette and Guérin's strain of *B. tuberculosis* (B.C.G.) has not caused tuberculosis in guinea-pigs even after an intensive course of 5 doses of 20 mgm. each of the living vaccine.

(2) Guinea-pigs inoculated with B.C.G. and later with virulent *B. tuberculosis* (human or bovine strains) tend to live for longer periods than others which have received an infecting dose of virulent organisms only. This is particularly marked in Experiment 4, where the infecting dose was minimal; in this experiment complete protection was obtained in 27 % of animals.

(3) The vaccinated guinea-pigs either showed no recognizable signs of tubercular infection as in the case of 6 guinea-pigs in Experiment 4, or, in the case of guinea-pigs which died from the disease, the infection was generalized and similar to that of the controls.

(4) It is uncertain from the evidence here produced that B.C.G. is a much better experimental prophylactic than living attenuated bacilli, Koch's bacillary emulsion (B.E.), dead tubercle bacilli, or certain other vaccines which have been in vogue at various periods in the past.

We have to express our thanks to Prof. Calmette for his constant kindness throughout this work, in supplying the original cultures and in allowing us to consult him concerning the details of his experiments.

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THE TESTING AND STANDARDIZATION OF *B. WELCHII* (*PERFRINGENS*) ANTITOXIN.

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THE recent increase in the use of *B. welchii* (*B. perfringens*) antitoxin in intestinal surgery (Williams, 1926), puerperal sepsis following abortion (Nürnberger, 1925, Lehman, 1926, and Inkster and Okell, 1924), and infection of the gall-bladder (Gould and Whitby, 1927), has directed attention to the problem of standardization of the antitoxin.

METHODS OF TESTING.

It is not proposed here to review all the references to the demonstration of antitoxic power of *B. welchii* serum, or to deal with testing against living cultures, one of the methods used by Weinberg and others. In Table I is given a summary of the chief methods of testing toxin and antitoxin used in recent years. Bull (1917) used for the titration of serum intramuscular injection of pigeons. He chose first as the "test dose" one minimal lethal dose of the filtrate of a young broth culture of *B. welchii*. His provisional "unit" of antitoxin was the smallest amount which when mixed with the test-dose of toxin and injected completely protected the pigeon against the toxin.

At the Wellcome Physiological Research Laboratories (Medical Research Committee, 1919) the intramuscular injection into mice was used, and, of the toxins available at first, the test-dose was of necessity limited by volume to two fatal doses. It was found that one-half of the Bull unit protected against two fatal doses of the chosen toxin. The W.P.R.L. unit used provisionally in the war was therefore made equivalent to twice the amount of serum that would protect against two fatal doses of the toxin originally chosen. A batch of serum was chosen as a standard and titrated as closely as possible against this original toxin. Subsequent toxins were in their turn standardized against this standard serum, but only those toxins were retained for test purposes which contained at least two fatal doses in the test-dose.

Bengtson (1920) chose as the test-dose of toxin ten times the minimum dose lethal for the pigeon when given intramuscularly; this test-dose was contained in a volume of 2 c.c. to 2·5 c.c. of the toxin then available.

Intravenous injection into rabbits and mice has been practised by many workers in this field. It was used on the large scale for testing at these laboratories during the war; the method has been developed during the past

TABLE I.—*Methods of Testing Toxin.*

Pigeon	Intramuscular	M.L.D.	0·1 to 1·0 c.c.	Bull, 1917; Bengtson, 1920.
Mouse	„	„	„ „	Present authors, 1918–1928 (*M.R.C. Report*, 1919).
„	Intravenous	„	0·01 to 0·1 c.c.	Ditto.
Guinea-pig	Intracutaneous	M.R.D.	„ „	Klose, 1916; Glenny and Allen, 1921.
In vitro	Hæmolysin	M.H.D.	„ „	Henry, 1917; Weinberg, 1918.

TABLE II.—*Titration of Standard Serum Against Fixed Doses of Toxin.*

Dose of standard serum.	*In vitro.*	Mouse.		Guinea-pig.
	Hæmolysis.	Intravenous.	Intramuscular.	Intradermic.
0·0018	Complete	...	Died	...
0·0020	„	Died	„ Œdema	Necrosis. Slight necrosis.
0·0022	Nearly complete	„	Lived „	No necrosis.
0·0025	Slight hæmolysis	Lived „	„ „	...
0·003	No hæmolysis	„ „	„ „	...

TABLE III.—*Neutral Points.*

Standard serum	0·0025	0·0022	0·0020	0·002
Serum A	0·014	0·012	0·01	0·012
„ B	0·00065	0·0006	0·0006	0·00055–75
„ C	0·0021	0·002	0·0017	0·0016
„ D	0·001	0·00075	0·0007	0·0008

Calculated Values.

(Standard = 45 units.)

Serum A	8	8	9	8
„ B	170	160	150	120–170
„ C	50	50	50	60
„ D	110	130	130	120

few years and much used by Dalling and Mason (1924), first in connection with "lamb dysentery cultures" (? *B. welchii*), and later with *B. welchii* (1926).

The intracutaneous method of testing in guinea-pigs mentioned by Klose (1916) was used by Glenny and Allen (1921), and has been much used during the present work. The technique of this method differs in no essential detail from that practised in testing diphtheria toxin and antitoxin; the lesion is necrotic, appears in twenty-four hours and quickly heals, but can be traced for some days later.

It is important to know to what extent the results of these methods of titration agree. Towards the end of the war, Glenny and Allen traced "primary and secondary response" of horses to injections of *B. welchii* toxin or toxin-antitoxin mixtures, using simultaneously for titration the intramuscular injection of mice and intracutaneous injection of guinea-pigs. The results given by the two methods showed fairly close agreement. About the same time in a limited number of experiments they found a close relation between the results of the intravenous and intramuscular methods of titration. This comparison of methods has been taken up again recently. A typical result is shown in Table II. It will be seen that there is a close agreement between the values given by the three methods. We have appended Table III (*v. infra* units of measurement), in which are shown the values of four different sera. For convenience a fixed dose of test toxin was used and the necessary calculations made for the intradermic method.

A considerable amount of further work will be necessary with numbers of different toxins from different strains and different antitoxins before it can be stated that the agreement holds under all circumstances; this and an inquiry into the toxin and toxoid relationships are in progress and the results will be published later.

In Table II is also shown the result of titration of the anti-hæmolytic property of the antitoxin. Throughout the literature much attention has been directed to the hæmolytic powers of *B. welchii* and its toxin. In recent years Weinberg (1918) and Henry (1922) have examined and titrated hæmolysin. Dalling, in his lamb dysentery work, investigated the relation of the hæmolysins and anti-hæmolysins connected with "lamb dysentery cultures" and *B. welchii*. More recently Mason, Glenny and Dalling (in preparation for publication) have found that there is apparently the close relationship shown in the table.

Our colleagues, Buttle and Trevan, kindly permit us to say that they have been investigating for some time past methods of "physiological standardization," using excised smooth muscle-tissue suspended in Ringer solution in the examination of toxins of the gas-gangrene anaerobes.

UNITS OF MEASUREMENT.

The provisional war units already referred to, established as they were against one or two fatal doses of toxin, served their purpose at the time. For the purpose of fixing a unit it would on general grounds be desirable to choose a test-dose of *B. welchii* toxin containing approximately 100 minimum lethal

doses if this amount were contained in a convenient volume of the toxin, as in the case of diphtheria, tetanus, *botulinus* and *œdematiens* toxins. The volume of 100 m.l.d. of *B. welchii* toxin would be inconveniently large; it is, however, possible to make use of 10 m.l.d. in the pigeon-intramuscular method and the mouse-intravenous method.

A toxin having been chosen whose test-dose is contained in a convenient volume and representing a reasonable number of lethal doses, it is next necessary to find its equivalent in standard serum; this now becomes the standard to which all future references are made. Whether this equivalent is termed one unit or some fraction of a unit is a matter for later consideration.

Bengtson determined the amount of a certain serum protecting against ten pigeon-intramuscular fatal doses of a certain toxin and called it 1/100th of a unit. The standard serum chosen by her contained 100 equivalents, and therefore 1 unit per c.c.

The method chosen by us was as follows:

A "toxin" was prepared, precipitated by ammonium sulphate, dried and ground; this material is reasonably stable, whereas the liquid toxin may rapidly deteriorate. A typical titration of such a toxin is shown in Table IV. Approximately ten times the mouse-intravenous fatal dose was accepted as a convenient "test dose" of toxin. A certain serum of low value was chosen as our standard serum. The volume of this serum which, when mixed with the test dose of toxin and injected into mice, protected half of a group of six or more mice, was determined in a series of tests; it proved to be 0·0022 c.c. A dose of serum about 10% higher, 0·0025 c.c., usually protected 90% or more of the mice, whereas a dose 10% lower, 0·0020 c.c., usually failed to protect 90% or more of the mice.

TABLE IV.—*Titration of Toxin.*

	Precipitated : Mice, intravenously.		
c.c. of toxin. 16 mgm. per c.c.			
0·03	+	+	+
0·025	+	+	L
0·02	+	+	L
0·015	L	L	L
0·01	L	L	

A second test toxin was titrated against the standard serum until a test dose was chosen which gave corresponding results with these doses of the test serum. This test dose of toxin was found to contain nearly 20 fatal doses. Thus it is seen that, as in the case of diphtheria and tetanus and other toxins, the number of fatal doses contained in the "test dose" of toxin is not constant, but depends on the relation between the amount of toxin and toxoid present.

Possibly the English Authority under the Therapeutic Substances Act, the U.S.A. Public Health Service and the League of Nations Standardization Committee will eventually decide on an official unit. Meanwhile we felt that

it was advisable to choose a unit of antitoxin such that the strength of an antitoxin expressed in "units" would be of the same order as that of the well-established sera such as those of diphtheria and tetanus. We considered, therefore, adopting as our unit ten times the amount of serum that protects against the originally chosen "test dose" of toxin; this would be contained in 0·022 c.c. of our standard serum. When we tested by the mouse-intravenous method a sample of the stock of antitoxin held at the Hygienic Laboratory, Washington, which Dr. McCoy kindly sent to us, we found that one "Bengtson unit" was equivalent to 120 of our units. To avoid numerical complication we are now altering our unit so that the relation between the "Bengtson unit" and the provisional unit we are adopting (the "W unit") is exactly 100 to 1, *i.e.* 0·01 c.c. of the Hygienic Laboratory serum is equal to our "W unit."

If the unit suggested by Bengston for *B. welchii* antitoxin were adopted, serum laboratories with horses producing many different antitoxins would find that the serum obtained from horses of high value would contain, in the case of diphtheria, 2000 units and upwards, for tetanus 2000 units and upwards, for *B. welchii*, on the other hand, 3 to 5 units; there would be a corresponding lack of relation in the figures used by the clinician in deciding on dosage. We feel that it would be of advantage to maintain as close a relation as practicable between the "dosage figures" which the clinician has to remember in prescribing treatment with sera. We therefore suggest that a unit 100 times smaller than the "Bengtson unit" would be the most convenient to use. There would appear to be no very sound reason why units of all antitoxin should be expressed in numbers containing many cyphers, and probably the "thousands" in the nomenclature of units of sera could be dropped with advantage, but if such a change is to be made, we would suggest that it would be advisable to make the change for all sera at one time. In Table V we have summarized the important points relating to the units of measurement.

TABLE V.—*Units of Measurement.*

			Minimal number of M.L.D. in test-dose.	Number of test-doses neutralized by one unit.	Expressed in "W" units.
Temporary war units:					
Bull	Pigeon	Intramuscular	1	1	$\frac{1}{10}$ to $\frac{1}{20}$
W.P.R.L.	Mouse	,,	2	2	
Modern units:					
Bengtson	Pigeon	Intramuscular	10	100	100
W.P.R.L.	Mouse	Intravenous	10	10	1

SUMMARY AND CONCLUSIONS.

1. *B. welchii* antitoxin has been titrated against precipitated toxin by intramuscular or intravenous injection into mice, intradermic injections into guinea-pigs, determination of anti-hæmolytic titre *in vitro*, or by means of excised smooth muscle-tissue.

2. All these methods are probably but different means of titrating the same principle since the values given agree fairly closely.

3. A provincial "unit" of antitoxin corresponding to 0·01 c.c. of the "Bengtson unit" serum has been adopted.

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